Dirk-Jan Slebos

Pathophysiology and airway inflammation in bronchiolitis obliterans after lung transplantation

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STELLINGEN
behorende bij het proefschrift
"Pathophysiology and airway inflammation
in Bronchiolitis Obliterans after lung transplantation"
van Dirk-Jan Slebos, november 2003.

1. Voor de beoordeling van de immunologische bronchoalveolaire lavage is het tijdstip na transplantatie van belang voor de interpretatie van de uitslag bij patiënten na longtransplantatie (dit proefschrift).

2. Na een longtransplantatie is een hoog percentage neutrofiele granulocyten bij een normaal totaal celaantal in de bronchoalveolaire lavage vloeistof zeer suggestief voor de aanwezigheid van bronchiolitis obliterans (dit proefschrift).

3. De aanwezigheid van elke eosinofiel in de bronchoalveolaire lavage vloeistof bij patiënten na longtransplantatie is voorspellend voor het ontstaan van bronchiolitis obliterans (dit proefschrift).

4. Vanaf een half jaar na longtransplantatie wegen de risico's en de belasting van het nemen van routine follow-up transbronchiale biopten voor een patiënt niet op tegen de zeer geringe therapeutische consequenties.

5. Verdere ontwikkeling van de immunologische bronchoalveolaire lavage zal in de toekomst een meer sensitief en specifiek diagnosticum kunnen opleveren dan de huidige uitkomst van het nemen van transbronchiale biopten.

6. Gezien de hoge morbiditeit en mortaliteit van patiënten na een longtransplantatie en de zeer toxische bijwerkingen van de gebruikte immunosuppressive medicatie lijkt het opportuun klinische studies te initiëren naar het therapeutische effect van haem oxygenase-1 en koolstof monoxide op het voorkomen van orgaanafstoting na longtransplantatie.

7. Het verplicht afsluiten van een 'mantelzorgcontract' bij opname zal niet alleen zorgen voor een grote kostenbesparing in de gezondheidszorg, maar zal er ook toe leiden dat de verantwoordelijkheid van de familie voor een patiënt niet ophoudt bij de ingang van het ziekenhuis.

8. "Zonder donorcodicil geen orgaantransplantatie" lijkt de juiste reactie op de daling van het aantal geregistreerde donorcodicil houders.

9. Het is de hoogste tijd dat de Nederlandse bevolking de bewering "De longarts leeft van de rook" niet meer zo letterlijk opvat.

10. Kantelende organisaties, kantelthema's en kantelmedewerkers: De gezondheidszorg hoeft echt niet om te vallen!

11. Om opnieuw een parlementaire enquête te voorkomen lijkt de meest voor de hand liggende oplossing voor de definitieve ontsluiting van het Noorden het liberaliseren van de maximumsnelheid op de snelwegen in de drie noordelijke provincies.

Rijksuniversiteit Groningen

Pathophysiology and airway inflammation in bronchiolitis obliterans after lung transplantation

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
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door

Dirk-Jan Slebos

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te Zwolle

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Paranimfen: drs. Joost C.H. van Oostrom
dr. Paul L. van Haelst

Wat is wijs

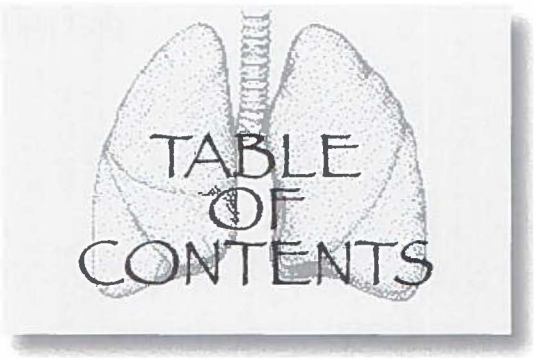
Heel dicht daar bij de oorsprong
daar neemt de eenvoud toe
complexiteit wordt minder
ver weg van 't menselijk gedoe

Heel dicht daar bij de kern
daar valt de bijzaak af
het scheidt zich van de hoofdzaak
het koren van het kaf

Heel dicht daar bij de oorsprong
daar zie je de natuur
daar is de kennis en de wijsheid
eenvoudig en heel puur

Je met die kern verbinden
hoe logischer het is
je voelt wel waar het heen moet
je voelt wat wijsheid is

Marleen, mei 2003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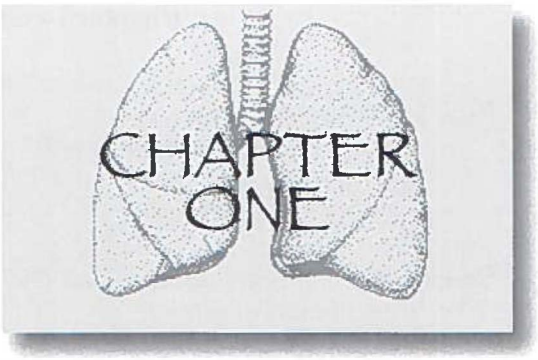


General introduction

INTRODUCTION

Lung transplantation (LTX) is the ultimate treatment modality for patients with end-stage lung- and/or pulmonary vascular disease due to various underlying disorders (table 1) [1-2]. Although the quality of life increases significantly after LTX, recipients remain in a continuous hazardous situation, which is best illustrated by a median survival time of 5 years (figure 1) [1,3]. The per- and post-operative mortality is high and already accounts for 10% of the loss of life in the first month after surgery.
Causes of death in this period constitute of severe reperfusion damage, cardiac failure, primary graft failure and infectious diseases [1]. But also after survival of the first months post LTX, serious morbidity threatens the recipient. Infections, renal failure, diabetes mellitus, induction of malignancies and chronic allograft rejection are all responsible for the poor survival [1,2,4].

CHRONIC ALLOGRAFT REJECTION

Chronic rejection of the lung allograft is defined as a fibrosing process of the lung, which primarily affects the smaller conducting airways and the pulmonary vasculature. The process involving the conducting smaller airways is 'bronchiolitis obliterans' (BO), while that affecting the pulmonary vasculature has been named graft atherosclerosis [6].

BO is the major long-term complication after LTX. According to the international registry 50% of all LTX recipients will have developed BO within five years after LTX (figure 2) [1]. BO and its infectious complications are responsible for serious morbidity and account for 50-70% of the mortality after LTX [1,5,7]. Once end-stage BO is present, re-transplantation is the only therapeutic option available [5,6].

RISK FACTORS FOR BO

At present, the exact pathophysiology of BO remains unknown. It is hypothesised that the development of BO involves epithelial damage due to repetitive injury of different origin, ultimately leading to irreversible airway obliteration [5,7]. This

	Groningen*	International registry**
COPD/Emphysema	45 (21.4%)	3462 (39.4%)
Interstitial pulmonary fibrosis	20 (9.5%)	1486 (16.9%)
Cystic Fibrosis	44 (20.9%)	1412 (16.1%)
Alpha-1 antitrypsin deficiency	54 (25.7%)	815 (9.3%)
Primary pulmonary hypertension	12 (5.7%)	401 (4.6%)
Sarcoidosis	2 (1.0%)	232 (2.6%)
Bronchiectasis	7 (3.3%)	190 (2.2%)
Re-lung transplantation	5 (2.3%)	156 (1.8%)
Congenital heart disease	10 (4.8%)	103 (1.2%)
Lymphangioleiomyomatosis	3 (1.4%)	95 (1.1%)
Connective tissue disorder	7 (3.3%)	38 (0.4%)
Cancer	0	35 (0.4%)
Other	5 (2.3%)	255 (2.9%)
Total	210	8781

TABLE 1.
*Pre- lung transplantation diagnoses and numbers of patients transplanted in *Groningen (1991-2002) and ** the total numbers of the international registry as collected by The International Society for Heart and Lung Transplantation (1995-2001) [1].*

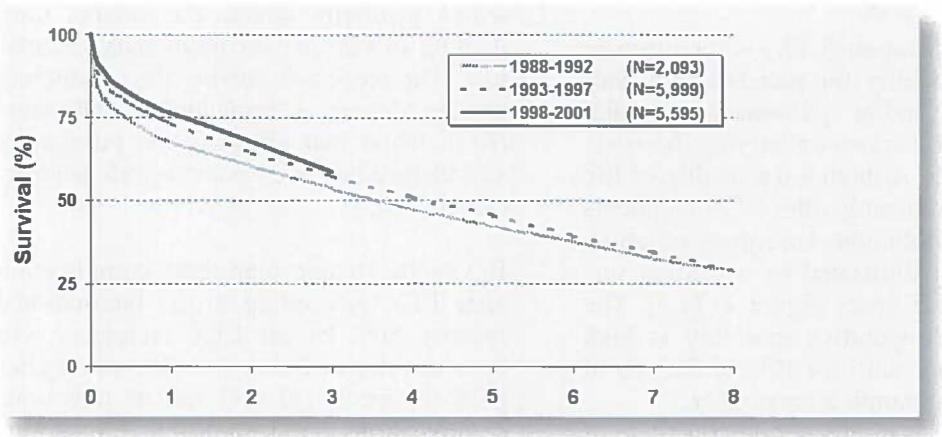


FIGURE 1.
Cumulative survival after lung transplantation given for three consecutive time periods. Numbers and figure extracted from the international registry [1].
The best survival occurs during the most recent time period (top line 1998-2001).

epithelial damage may occur as a result of a number of independent risk factors known to be linked to BO development, such as allograft reactivity (including HLA-DR mismatch), CMV infections, acute and chronic bacterial infections, number of acute rejection episodes, graft ischaemic time, donor age, chronic aspiration and possibly also the maintenance immunosuppressive regime used (see figure 3 for detailed hypothesised mechanisms) [5,8-10].

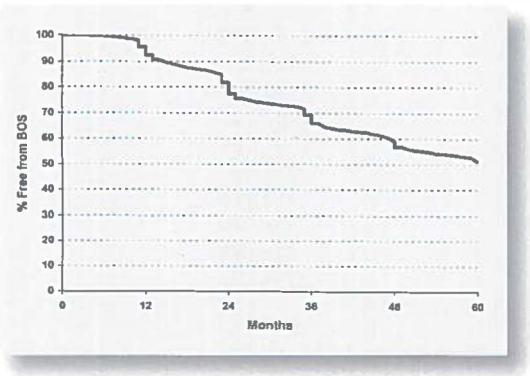


FIGURE 2.
Cumulative freedom of Bronchiolitis Obliterans Syndrome (BOS) after lung transplantation. Numbers and figure extracted from the international registry [1].

PATHOGENESIS OF BO

BO has two distinct histopathologically recognisable phases, starting with an alloimmune phase with lymphocyte infiltration of the bronchiolar structures followed by a chronic fibroproliferative phase. In general, the current data available supports a cellular immune response in the development of BO, but it is still a subject of debate which T-lymphocyte subtype dominates BO [5,6]. Several reports published, using the mouse-tracheal allograft model for the study of chronic allograft rejection report either the dominance of only a cytotoxic lymphocyte subtype population or both T-helper and cytotoxic lymphocytes [11,12]. Human studies show opposing results as well. Both proliferation of the $CD4^+$ T-cell population and a dominance of $CD8^+$ T-cells have been reported [13,14]. Even both elevated and decreased CD4/CD8 ratios have been reported with respect to the presence of BO [14,15].

Initially, these T-lymphocytes infiltrate in the airways in response to inflammation and epithelial injury, leading to epithelial cell necrosis (secondary to the proposed BO risk factors). When confluent areas of

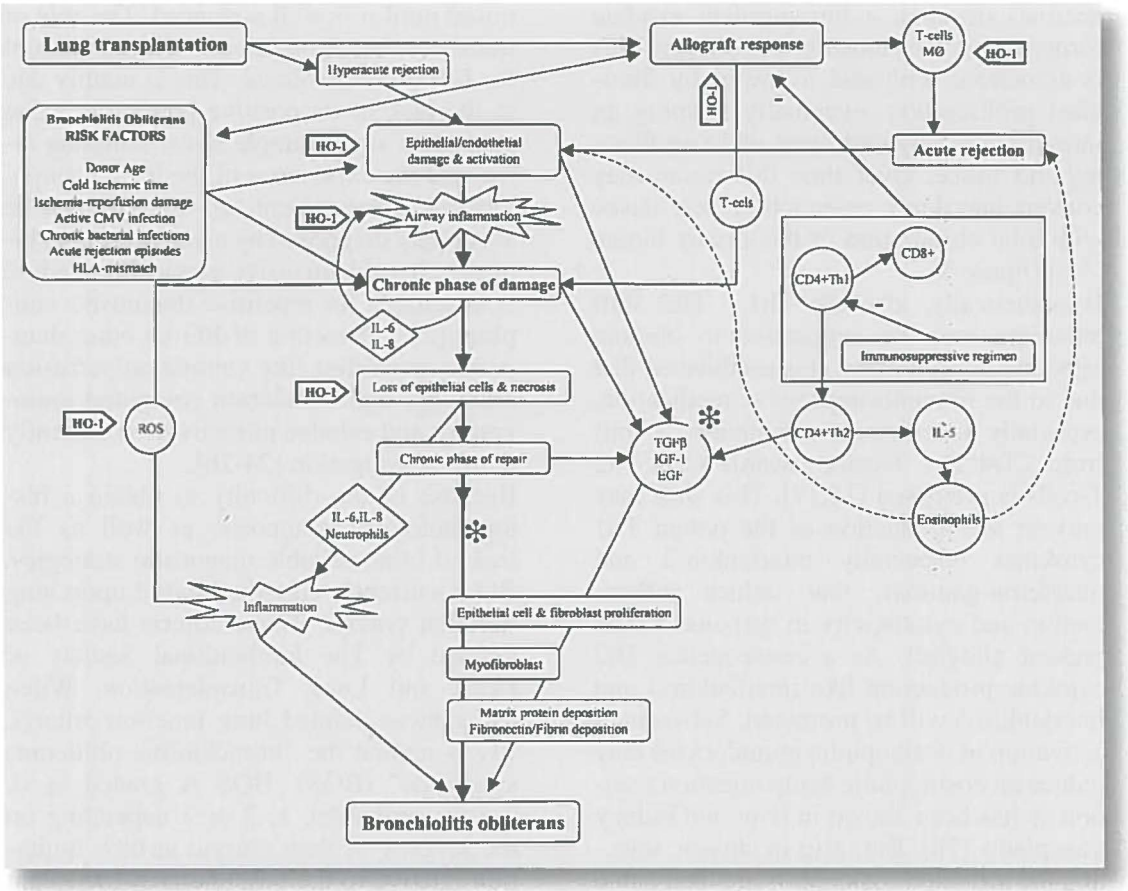


FIGURE 3.

Schematic presentation of mechanisms possibly involved in the development of bronchiolitis obliterans after lung transplantation. The development of bronchiolitis obliterans (BO) starts immediately after transplantation, when respiratory epithelial and endothelial cells become damaged due to cold-ischemia, ischemia-reperfusion damage and the hyperacute allograft response. After this first injury, infectious agents and recurrent acute rejection episodes continue to damage the epithelial cells. The epithelial and endothelial cell damage present results in an inflammatory response that leads to a more chronic phase of epithelial and endothelial cell damage. Persistent injury and inflammation will lead to epithelial cell necrosis, which will induce a chronic phase of repair. These activated epithelial cells are able to produce both pro-inflammatory cytokines and growth factors. The cytokines support an ongoing, mostly neutrophilic, inflammatory response causing a subsequent release of reactive oxygen species (ROS). These ROS also support the chronic process of epithelial damage ultimately leading to BO. The growth factors produced by the activated epithelial cells induce epithelial cell and fibroblast proliferation and due to an overwhelming repair reaction BO develops. In this response, natural genetic variability (polymorphisms) of various factors (e.g. TGFβ) may account for a lesser or stronger repair. The immunosuppressive regimen promotes a shift from CD4⁺Th1 cells to proliferation of CD4⁺Th2 cells. These regulatory T-cells in turn produce both pro-fibrotic cytokines, but may also activate eosinophils. These activated eosinophils can directly induce an acute rejection and by the release of toxic mediators (major basic protein, eosinophil cationic proteins, eosinophil-derived neurotoxin) and tryptase from activated mast cells, induce a chronic tissue damaging reaction. ->: Arrows indicate stimulation; ⊐ indicates sites of potential inhibiting action by heme oxygenase-1 (HO-1) and downstream products e.g. carbon monoxide and biliverdin; ✻ indicates possible role for genetic factors: TGF-β and HO-1 polymorphisms; IL: interleukin; INFγ: interferon gamma; TGFβ: tissue growth factor-beta; IGF-1: insulin-like growth factor-1; EGF: epithelial growth factor; MØ: alveolar macrophages.

necrosis develop, a fibropurulent exudate forms within the lumen of the airway. This is associated with and followed by fibroblast proliferation, eventually forming an intraluminal polypoid mass of loose fibromyxoid tissue. Over time this tissue may convert into dense eosinophilic scar tissue with total obliteration of the airway lumen [5,6] (figure 3).

Hypothetically, also the Th1 - Th2 shift paradigm may be supportive to chronic rejection development. It is established that due to the immunosuppressive medication, especially the calcineurin inhibitors, a shift from $CD4^+$Th1 T-cells towards $CD4^+$Th2 T-cells is promoted [16,17]. This shift may prevent the production of the potent Th1 cytokines (especially interleukin-2 and interferon-gamma), that induce inflammation and cytotoxicity in response to the present allograft. As a consequence Th2 cytokine production like interleukin-4 and interleukin-5 will be promoted. Subsequent activation of eosinophilic granulocytes may induce an eosinophilic acute rejection reaction as has been shown in liver and kidney transplants [18]. But, also in chronic rejection the activated eosinophils are likely able to contribute to the fibrosing process due to the production of major basic protein and subsequent tryptase release from activated mast cells. The other consequence of the Th1 - Th2 shift is the production of growth factors such as tissue growth factor-β and insulin-like growth factor-1. These growth factors can induce an overwhelming repair mechanism in response to the epithelial damage that in turn will lead to a pro-fibrotic reaction that promotes the development of BO. Once BO is present, high IL-8 cytokine levels and neutrophilic inflammation dominate the airways [16-19] (figure 3).

DIAGNOSIS OF BO

The development of BO after LTX is the most common cause of late graft failure but may not be histopathologically diagnosed until it is well advanced. The role of transbronchial biopsies as a diagnostic tool for BO is controversial. This is mainly due to the lack of its positive predictive value caused by small sample sizes, sampling error, and the experience of the local histopathological department [20-22]. BO can be accurately diagnosed by using open lung biopsies, but this invasive surgical procedure is unsuitable for repetitive diagnostic sampling [23]. Detection of BO by other diagnostic modalities like ventilation/perfusion scanning, high resolution computed tomography, and exhaled nitric oxide is currently under investigation [24-26].

Because of the difficulty to obtain a histopathological diagnosis, as well as the lack of other reliable diagnostic strategies, BO is currently classified based upon lung function criteria. These criteria have been defined by The International Society of Heart and Lung Transplantation. When using these defined lung function criteria, BO is named the "bronchiolitis obliterans syndrome" (BOS). BOS is graded as 0, 0-p (p: probable), 1, 2 or 3 depending on the severity of their current airflow limitation relative to the baseline post-transplant FEV_1 and FEF_{25-75} (table 2) [27]. Although impaired lung function may signify BOS, it is likely that considerable irreversible damage is already present in the airways of the lung allograft before this measurable reduction in lung function. Identification of these early changes seems important since early therapeutic intervention can prevent or slow down the natural course of the developing chronic graft failure [28].

BRONCHOALVEOLAR LAVAGE IN BO

Local airway inflammation in patients with pulmonary diseases can be detected by a number of diagnostic modalities. Bronchoalveolar lavage (BAL), endobronchial biopsies, sputum induction, exhaled breath condensate and exhaled air examination

are all often used for assessment of airway inflammatory characteristics [29,30]. In this thesis we have examined the airways in patients after LTX by using bronchoalveolar lavage fluid (BALF) examination (figure 4). BALF sampling is easily performed during the routine follow up bronchoscopies after LTX. The BALF procedure is an established diagnostic tool for microbiological surveillance [31]. Furthermore, immunological BALF analysis is used to investigate local airway changes after LTX, as well as airway changes during acute rejection and BO [32-35]. Because it is thought that the predominant lesions in BO are located in the small airways, we choose to analyse both a bronchial fraction, which is assumed to represent the proximal airways, and an alveolar fraction, reflecting the peripheral airways [36] (figure 4).

In manifest BO, BALF analysis is characterised by a marked airway neutrophilia and

BOS 0	FEV_1 > 90% of baseline and FEF_{25-75} > 75% of baseline	BOS 0 *(original classification[3])*
BOS 0-p	FEV_1 81% to 90% of baseline and/or FEF_{25-75} ≤ 75% of baseline	
BOS 1	FEV_1 66% to 80% of baseline	
BOS 2	FEV_1 51% to 65 % of baseline	
BOS 3	FEV_1 < 50% of baseline	

TABLE 2.
The in 2002 revised BOS (bronchiolitis obliterans syndrome) criteria. FEV_1: forced expiratory volume in one second. FEF_{25-75}: maximal mid-expiratory flow rate (over the middle half of the forced vital capacity manoeuvre) [18].

Compound measured in BAL	in BO*	Reference
Neutrophils	↑	[19,30,35,37-41]
Interleukin-1	=	[42]
Interleukin-1 receptor antagonist	↑	[42]
Interleukin-8	↑	[19,35,37,41]
Interleukin-10	=	[40,41]
Tumor necrosis factor-α	=	[40,41]
Tissue growth factor-β	=/↑	[40,41]
Insuline-like growth factor-1	↑	[42]
Monocyte chemoattractant protein-1	↑	[37, 43]
Myeloperoxidase	↑	[39]
Neutrophil elastase	↑	[44]
Eosinophil cationic protein	↑	[39]
RANTES	↑	[37]
Soluble intercellular cell adhesion molecule-1	=	[37]
Vascular cell adhesion molecule-1	=	[37]
Vascular endothelial growth factor	↓	[45]

TABLE 3.
*Overview of the available literature on diverse compounds measured in BALF samples from patients with proven and/or future bronchiolitis obliterans. RANTES: 'regulated upon activation, normal T-cell expressed and secreted'; * ↑: Indicates elevated levels of the protein measured in BALF when BO is present; =: indicates not elevated when BO is present; ↓ indicates lower when BO is present.*

high IL-8 levels [14,19,30,35,37-39]. Besides these consistent findings, numerous small studies on BALF have analysed proteins possibly linked to the development of BO (see table 3 for a detailed description of all measured compounds in BALF sampled from patients with BO after LTX) [14,19,30,35-45]. All together these studies support the multi-factorial pathogenesis of BO, but it is difficult to draw conclusions from each individual study with respect to BO development.

TREATMENT AND PREVENTION OF BO

Current treatment modalities for BO implicate adjustment of maintenance immunosuppressive regimens e.g. changing cyclosporine to tacrolimus or sirolimus and/or changing azathioprine to mycophenolate mofetil [5,46-48]. Furthermore, "rescue therapy" strategies are applied when the adjustment of the maintenance immunosuppressive regimen has proven ineffective. These strategies involve admi-

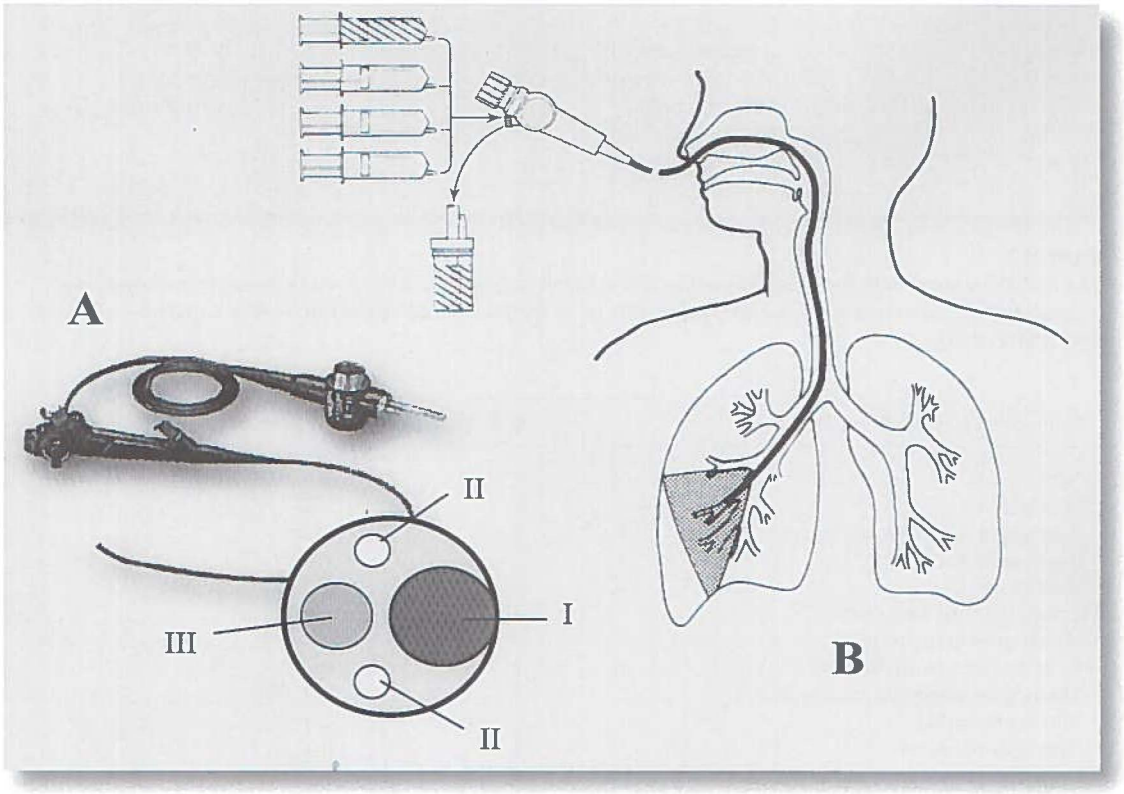


FIGURE 4A AND B.
A: Flexible bronchoscope. Schematic drawing showing the tip of the bronchoscope when held in normal working position. I: working channel; II: light source; III: camera. B: Immunological bronchoalveolar lavage procedure as used in the Groningen University Hospital: 1. The tip of the bronchoscope is positioned in near-wedge of the segmental bronchus selected. 2. The bronchoscopist should face the left-hand bronchial wall, facilitating suction (minus 10-20 cm water pressure). 3. The first aliquot of pre-warmed (37°) 20 ml phosphate buffered saline is sent for microbiological examination. The second pre-warmed 20 ml portion (considered the bronchial fraction) is isolated before three aliquots of pre-warmed 50 ml are pooled (the actual alveolar fraction). Both bronchial and alveolar fractions are thereafter immediately placed on ice for further examination [72].

nistration of methylprednisolone, different types of antilymphocyte antibodies, methotrexate, inhaled cyclosporine, total body irradiation, extracorporal photopheresis, and fundoplication [5,49-55]. But all have been shown to be limitedly effective. Yet these strategies are the best available, but the side effects, recurrent reactivation of BO, local availability and lack of scientific support (all studies performed are small and descriptive or uncontrolled trials with historical controls) limits all these therapies to an experimental level.
All these therapeutic strategies may eventually achieve slower progression of BO, or even stabilisation of the process. However, so far no agent is available to cure BO [56]. Therefore, preventive strategies with respect to development of BO seem to be the most effective in the 'treatment' of BO. Preventive strategies imply prevention of the proposed risk factors e.g. prevention of cold ischemia, reduction of ischemia-reperfusion damage, prevention of acute rejection episodes and administration of CMV and bacterial prophylaxis [5, 9, 28, 57].

In conclusion, to date treatment of BOS is infrequently successful. Better understanding of the pathophysiology and earlier recognition of BO may result in improved long-term patient and graft survival.

A NOVEL IMMUNOSUPPRESSIVE STRATEGY AFTER LUNG TRANSPLANTATION: THE HEME OXYGENASE-1 AND CARBON MONOXIDE SYSTEM.

In recent years evidence on the cytoprotective role of heme oxygenase-1 (HO-1) and carbon monoxide (CO) has been accumulating *in vitro* and *in vivo* about. HO-1 can interfere in numerous physiological processes and has been shown to inhibit inflammatory responses, to slow down apoptosis and tumour growth, to protect against oxidative stress and to prevent allograft rejection after organ transplantation. HO-1 is the rate-limiting enzyme in the conversion of heme to iron, bilirubin and CO. Upregulation of HO-1 (by stimuli such as hypoxia, nitric oxide, lipopolysaccharides or reactive oxygen species) results in all these protective effects. The precise mechanism by which HO-1 acts is poorly understood. Remarkably most of these protective effects of HO-1 remain present both *in vitro* and *in vivo*, when HO-1 is inhibited and only CO is administered at very low, non-toxic, concentrations. This suggests that CO is the active key mediator (see figure 4 for a detailed description of the functional consequences of HO-1 and CO activity) [58-62].
To date, limited data are available on the role of HO-1 and CO in LTX allograft rejection. Recently, an increased expression of HO-1 in alveolar macrophages was observed in human LTX recipients with allograft rejection, suggesting a potential role of HO-1 in LTX [63]. In animal transplantation models, like a rat liver transplant model and in transplanted pancreatic island cells, allograft protection is achieved due to up-regulation of HO-1 [64,65]. Also in cardiac xeno-transplantation (mouse-to-rat) graft protection by both HO-1 modulation and/or CO administration has been shown [66,67]. HO-1 upregulation can furthermore induce cellular protection against ischemia/reperfusion (I/R) injury after reimplantation of the organ as has been shown in both a guinea pig LTX model and in a rat liver transplantation model [68,69]. The I/R injury is thereby possibly prevented by suppression of Th1 cytokines and a decreased apoptosis after reperfusion [69]. Recently CO itself has been shown to inhibit apoptosis in I/R lung injury by modulation of p38 MAP-kinase and caspase-3 [70].

Thus, knowing that the HO-1/CO system can inhibit allograft rejection and prevent I/R damage in animal models, it can be hypothesised that HO-1 and CO have a poten-

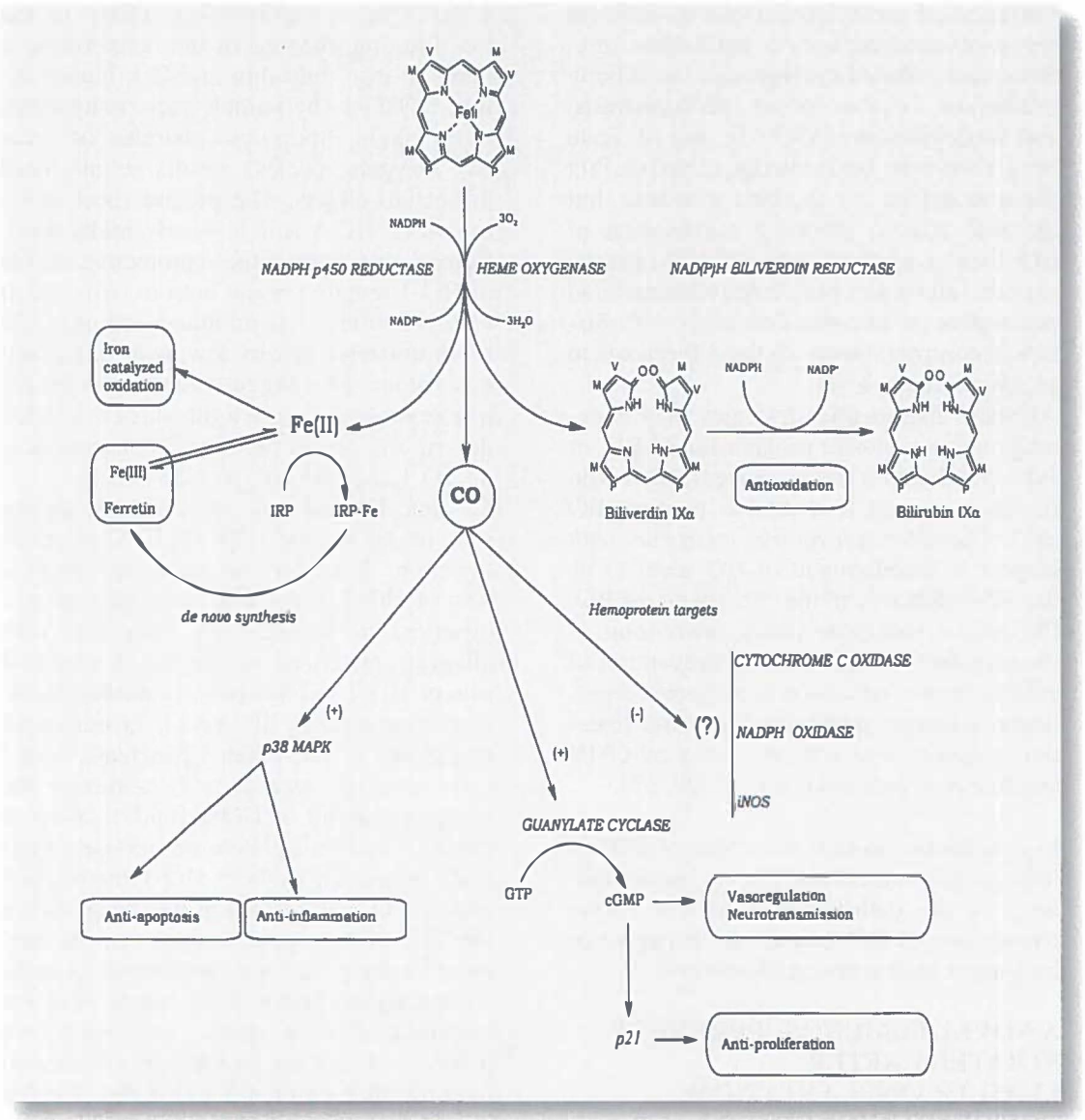


FIGURE 5.

Schematic presentation of the functional consequences of heme metabolism and CO production (adapted from ref. 44). Heme oxygenase (HO) degrades heme to biliverdin-IXα, carbon monoxide (CO), and iron. Biliverdin-IXα is converted to bilirubin-IXα by NAD(P)H biliverdin reductase. Both bile pigments have potent in vitro antioxidant activity. Redox-active iron released from HO activity may promote oxidative damage. However, by inactivating iron regulatory protein (IRP) activity, iron stimulates the synthesis of ferritin, an iron-sequestration protein and possible cytoprotectant. CO derived from the HO reaction has possible significance in the regulation of vascular and neural functions. The stimulation of cGMP-dependent signal transduction pathways may account for the vasodilatory and anti-proliferative effects. CO has potent anti-inflammatory effects, which depend on downregulation of pro-inflammatory cytokine production mediated by modulation of p38 MAPK. cGMP = guanosine 3',5'-cyclic monophosphate; Fe(II) = ferrous iron; Fe (III) = ferric iron; GTP = guanosine triphosphate; NOS = nitric oxide synthase; p38 MAPK = p38 mitogen activated protein kinase.

tial important protective role in preserving allograft function both directly after LTX as well as on the longer run.

AIM OF THE THESIS

Bronchiolitis obliterans accounts for serious morbidity and mortality after lung transplantation. Until now there is limited knowledge of the pathophysiology, and no sensitive and specific diagnostic modalities are available to accurately assess BO. Moreover, the current available treatment modalities fail to prevent or even stabilise bronchiolitis obliterans.

In **Part I** of this thesis we set out to investigate both the normal airway changes after lung transplantation as well as changes due to bronchiolitis obliterans. Until now it is not known which airway cellular changes occur after lung transplantation. Therefore, we examined in **chapter 2** the normal profile of bronchoalveolar lavage cell characteristics in stable patients with a good outcome after lung transplantation.
In **chapter 3** the bronchoalveolar lavage fluid characteristics in patients with acute and chronic allograft rejection are evaluated and tested for their diagnostic feasibility. Thereafter we examined in **chapter 4** the airway changes in bronchiolitis obliterans as well as acute rejection to determine the predictive value of bronchoalveolar lavage fluid examination with respect to the development of bronchiolitis obliterans. Due to the introduction of new immunosuppressive agents, with potential better treatment results, we examined in **chapter 5** the influence of different treatment modalities on the airways.
In **chapter 6** we describe the clinical utility of the immunological bronchoalveolar lavage procedure in a case-report, and finally in **chapter 7** we investigate the feasibility of sputum induction as a diagnostic tool to evaluate airway cellular and soluble compounds in lung transplant recipients.

In **part II** of this thesis, we propose a new concept of immunosuppression and graft protection, by reviewing (**chapter 8**) the potential of the heme oxygenase-1 and carbon monoxide system with respect to lung transplantation. To investigate a possible role for heme oxygenase-1 and carbon monoxide in lung transplantation, we examine the levels of exhaled carbon monoxide with respect to bronchiolitis obliterans in **chapter 9** and the heme oxygenase-1 expression in bronchoalveolar lavage fluid alveolar macrophages of lung transplant recipients in **chapter 10**.

Finally, **chapter 11** summarises this thesis and provides future perspectives with respect to further research and treatment options.

REFERENCES

1.Hertz MI, Taylor DO, Trulock EP, Boucek MM, Mohacsi PJ, Edwards LB, Keck BM. The registry of the international society for heart and lung transplantation: nineteenth official report-2002. J Heart Lung Transplant. 2002;21:950-70.
2. Arcasoy SM, Kotloff RM. Lung transplantation. N Engl J Med. 1999;340 :1081-91.
3. TenVergert EM, Essink-Bot ML, Geertsma A, van Enckevort PJ, de Boer WJ, van der Bij W. The effect of lung transplantation on health-related quality of life: a longitudinal study. Chest. 1998;113:358-64.
4. Vermeulen KM, Ouwens JP, van der Bij W, de Boer WJ, Koeter GH, TenVergert EM. Long-term quality of life in patients surviving at least 55 months after lung transplantation. Gen Hosp Psychiatry. 2003;25:95-102.
5. Estenne M,.Hertz MI. Bronchiolitis obliterans after human lung transplantation. Am J Respir Crit Care Med 2002;166:440-444
6. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Tazelaar HD. Revision of the 1990 Working Formulation for the Classification of Pulmonary Allograft Rejection: Lung Rejection Study Group. J Heart Lung Transplant. 1996;15:1-15.

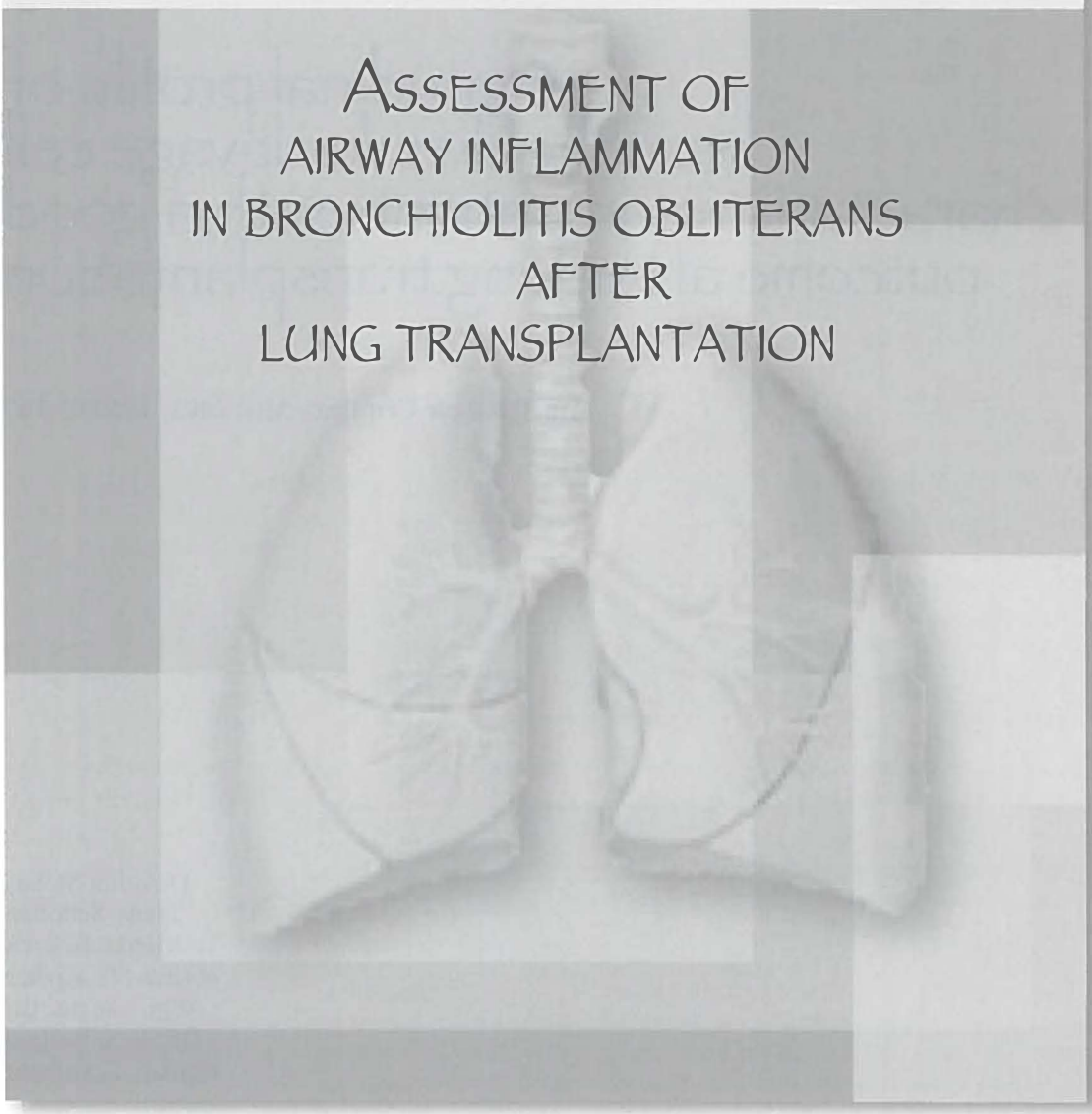
7. van Den Berg JW, Geertsma A, van Der Bij W, Koëter GH, de Boer WJ, Postma DS, ten Vergert EM. Bronchiolitis obliterans syndrome after lung transplantation and health-related quality of life. Am J Respir Crit Care Med. 2000;161:1937-41.
8. van den Berg JW, Hepkema BG, Geertsma A, Koeter GH, Postma DS, de Boer WJ, Lems SP, van der Bij W. Long-term outcome of lung transplantation is predicted by the number of HLA-DR mismatches. Transplantation. 2001;71:368-73.
9. Sharples LD, McNeil K, Stewart S, Wallwork J. Risk factors for bronchiolitis obliterans: a systematic review of recent publications. J Heart Lung Transplant. 2002;21:271-81.
10. Borger P, Kauffman HF, Scholma J, Timmerman JAB, Koëter GH. Human allogenic $CD2^+$ lymphocytes activate airway derived epithelial cells to produce Il-6 and IL-8. Possible role for the epithelium in chronic allograft rejection. J Heart Lung Transplant 2002;21:567-575
11. Neuringer IP, Walsh SP, Mannon RB, Gabriel S, Aris RM. Enhanced T cell cytokine gene expression in mouse airway obliterative bronchiolitis. Transplantation. 2000;69;399-405.
12. Neuringer IP, Mannon RB, Coffman TM, Parsons M, Burns K, Yankaskas JR, Aris RM. Immune cells in a mouse airway model of obliterative bronchiolitis. Am J Respir Cell Mol Biol. 1998;19: 379-86.
13. Duncan SR, Leonard C, Theodore J, Lega M, Girgis RE, Rosen GD, Theofilopoulos AN. Oligoclonal $CD4(^+)$ T cell expansions in lung transplant recipients with obliterative bronchiolitis. Am J Respir Crit Care Med. 2002;165:1439-44.
14. Ward C, Whitford H, Snell G, Bao H, Zheng L, Reid D, Williams TJ, Walters EH. Bronchoalveolar lavage macrophage and lymphocyte phenotypes in lung transplant recipients. J Heart Lung Transplant. 2001;20:1064-74.
15. Reynaud-Gaubert M, Thomas P, Gregoire R, Badier M, Cau P, Sampol J, Giudicelli R, Fuentes P. Clinical utility of bronchoalveolar lavage cell phenotype analyses in the postoperative monitoring of lung transplant recipients. Eur J Cardiothorac Surg. 2002;2160-6.
16. Shirwan H. Chronic allograft rejection. Do the Th2 cells preferentially induced by indirect alloantigen recognition play a dominant role? 1999. Transplantation;68:715-26
17. Holzknecht ZE, Platt JL. 2000. The fine cytokine line between graft acceptance and rejection. 2000. Nat Med. 6:497-8.
18. Goldman M, Le Moine A, Braun M, Flamand V, Abramovicz D. A role for eosinophils in transplant rejection. TRENDS in Immunology. 2001;22:247-251.
19. DiGiovine B, Lynch III JP, Martinez FJ, Flint A, Whyte RI, Iannettoni MD, Arenberg DA, Burdick MD, Glass MC, Wilke CA, Morris SB, Kunkel SL, Strieter RM. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. J Immunol 1996;157: 4194-4202.
20. Chamberlain D, Maurer J, Chaparro C, Idolor L. Evaluation of transbronchial lung biopsy specimens in the diagnosis of bronchiolitis obliterans after lung transplantation. J Heart Lung Transplant. 1994;13: 963-71.
21. Kramer MR, Stoehr C, Whang JL, Berry GJ, Sibley R, Marshall SE, Patterson GM, Starnes VA, Theodore J. The diagnosis of obliterative bronchiolitis after heart-lung and lung transplantation: low yield of transbronchial lung biopsy. J Heart Lung Transplant. 1993;12:675-81.
22. Pomerance A, Madden B, Burke MM, Yacoub MH. Transbronchial biopsy in heart and lung transplantation: clinicopathologic correlations. J Heart Lung Transplant. 1995;14:761-73.
23. Weill D, McGiffin DC, Zorn GL Jr, Alexander CB, Early LJ, Kirklin JK, Young KR. The utility of open lung biopsy following lung transplantation. J Heart Lung Transplant. 2000;19:852-7.
24. Siegel MJ, Bhalla S, Gutierrez FR, Hildebolt C, Sweet S. Post-lung transplantation bronchiolitis obliterans syndrome: usefulness of expiratory thin-section CT for diagnosis. Radiology. 2001;220: 455-62.
25. Ikonen T, Kivisaari L, Harjula AL, Lehtola A, Heikkila L, Kinnula VL, Kyosola K, Savola J, Sipponen J, Verkkala K, Mattila SP. Value of high-resolution computed tomography in routine evaluation of lung transplantation recipients during development of bronchiolitis obliterans syndrome. J Heart Lung Transplant. 1996;15:587-95.
26. Gabbay E, Walters EH, Orsida B, Whitford H, Ward C, Kotsimbos TC et al. Post-lung transplant bronchiolitis obliterans syndrome (BOS) is characterized by increased exhaled nitric oxide levels and epithelial inducible nitric oxide synthase. Am J Respir Crit Care Med 2000;162:2182-87.
27. Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M et al. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002; 21:297-310.
28. Revell MP, Lewis ME, Llewellyn-Jones CG, Wilson IC, Bonser RS. Conservation of small-airway function by tacrolimus/cyclosporine conversion in the management of bronchiolitis obliterans following lung transplantation. J Heart Lung Transplant 2000;19:1219-1223.
29. Kharitonov SA, Barnes PJ. Exhaled markers of inflammation. Curr Opin Allergy Clin Immunol. 2001;1:217-24.
30. Ward C, Snell GÍ, Zheng L, Orsida B, Whitford H, Williams TJ, Walters EH. Endobronchial biopsy and bronchoalveolar lavage in stable lung transplant recipients and chronic rejection. Am J Respir Crit Care Med 1998;158:84-91

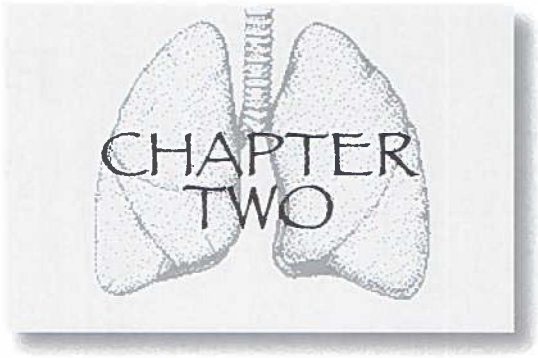
31. Chan CC, Abi-Saleh WJ, Arroliga AC, Stillwell PC, Kirby ThJ, Gordon SM, Petras RE, Metha AC. Diagnostic yield and therapeutic impact of flexible bronchoscopy in lung transplant recipients. J Heart Lung Transplant. 1996;15:196-205.
32. Tiroke AH, Bewig B, Haverich A. Bronchoalveolar lavage in lung transplantation. State of the art. Clin Transplant. 1999;13:131-157.
33. Clelland C, Higenbottam T, Stewart S, Otulana B, Wreghitt T, Gray J, Scott J, and Wallwork J. Bronchoalveolar lavage and transbronchial biopsy during acute rejection and infection in heart-lung transplant patients. Am Rev Respir Dis 1993;147: 1386-1392
34. Riise GC, Kjellstrom C, Ryd W, Schersten H, Nilsson FN, Martensson G, Andersson BA. Inflammatory cells and activation markers in BAL during acute rejection and infection in lung transplant recipients: a prospective, longitudinal study. Eur Respir J. 1997;10:1742-1746.
35. Zheng L, Walters EH, Ward C, Wang N, Orsida B, Whitford H, Williams TJ, Kotsimbos T, Snell GI. Airway neutrophilia in stable and bronchiolitis obliterans syndrome patients following lung transplantation. Thorax. 2000;55:53-59.
36. Kelly CA, Kotre CJ, Ward C, Hendrick DJ, Walthers EH. 1987. Anatomical distribution of bronchoalveolar lavage fluid as assessed by digital subtraction radiography. Thorax 42:624-628.
37. Reynaud-Gaubert M, Marin V, Thirion X, Farnarier C, Thomas P, Badier M,Bongrand P, Giudicelli R, Fuentes P. Upregulation of chemokines in bronchoalveolar lavage fluid as a predictive marker of post-transplant airway obliteration. J Heart Lung Transplant. 2002 Jul;21(7):721-30.
38. Clelland C, Higenbottam T, Stewart S, Otulana B, Wreghitt T, Gray J, Scott J, and Wallwork J. Bronchoalveolar lavage and transbronchial biopsy during acute rejection and infection in heart-lung transplant patients. Am Rev Respir Dis 1993;147: 1386-1392
39. Riise GC, Andersson BA, Kjellstrom C, Martensson G, Nilsson FN, Ryd W, Schersten H. Persistent high BAL fluid granulocyte activation marker levels as early indicators of bronchiolitis obliterans after lung transplant. Eur Respir J. 1999 Nov;14(5): 1123-30.
40. Belperio JA, DiGiovine B, Keane MP, Burdick MD, Ying Xue Y, Ross DJ, Lynch JP 3rd, Kunkel SL, Strieter RM. Interleukin-1 receptor antagonist as a biomarker for bronchiolitis obliterans syndrome in lung transplant recipients. Transplantation. 2002 Feb 27;73(4):591-9.
41. Elssner A, Jaumann F, Dobmann S, Behr J, Schwaiblmair M, Reichenspurner H, Furst H, Briegel J, Vogelmeier C. Elevated levels of interleukin-8 and transforming growth factor-beta in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells. Munich Lung Transplant Group. Transplantation. 2000 Jul 27;70(2):362-7.
42. Charpin JM, Stern M, Grenet D, Israel-Biet D. Insulinlike growth factor-1 in lung transplants with obliterative bronchiolitis. Am J Respir Crit Care Med. 2000 Jun;161(6):1991-8.
43. Belperio JA, Keane MP, Burdick MD, Lynch JP 3rd, Xue YY, Berlin A, Ross DJ, Kunkel SL, Charo IF, Strieter RM. Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome. J Clin Invest. 2001 Aug;108(4):547-56.
44. Scott JP, Holt DW, Wallwork J. Neutrophil elastase and obliterative bronchiolitis. Transpl Int. 1994;7 Suppl 1:S402-3.
45. Meyer KC, Cardoni AL, Xiang Z, Cornwell RD, Love RB. Vascular endothelial growth factor in human lung transplantation. Chest. 2001 Jan;119(1): 137-43.
46. Sarahrudi K, Carretta A, Wisser W, Senbaklavaci O, Ploner M, Neuhauser P, Dobrovits M, Miwai Marta G, Papp A, Klepetko W. The value of switching from cyclosporine to tacrolimus in the treatment of refractory acute rejection and obliterative bronchiolitis after lung transplantation. Transpl Int. 2002;15:24-8.
47. Ross DJ, Lewis MI, Kramer M, Vo A, Kass RM. FK 506 'rescue' immunosuppression for obliterative bronchiolitis after lung transplantation. Chest. 1997;112:1175-9.
48. Whyte RI, Rossi SJ, Mulligan MS, Florn R, Baker L, Gupta S, Martinez FJ, Lynch JP 3rd. Mycophenolate mofetil for obliterative bronchiolitis syndrome after lung transplantation. Ann Thorac Surg. 1997;64:945-8.
49. Dusmet M, Maurer J, Winton T, Kesten S. Methotrexate can halt the progression of bronchiolitis obliterans syndrome in lung transplant recipients. J Heart Lung Transplant. 1996;15:948-54.
50. Iacono AT, Keenan RJ, Duncan SR, Smaldone GC, Dauber JH, Paradis IL, Ohori NP, Grgurich WF, Burckart GJ, Zeevi A, Delgado E, O'Riordan TG, Zendarsky MM, Yousem SA, Griffith BP. Aerosolized cyclosporine in lung recipients with refractory chronic rejection. Am J Respir Crit Care Med. 1996;153:1451-5.
51. Kesten S, Rajagopalan N, Maurer J. Cytolytic therapy for the treatment of bronchiolitis obliterans syndrome following lung transplantation. Transplantation. 1996;61:427-30.
52. Snell GI, Esmore DS, Williams TJ. Cytolytic therapy for the bronchiolitis obliterans syndrome complicating lung transplantation. Chest. 1996;109: 874-8.
53. Ross DJ, Jordan SC, Nathan SD, Kass RM, Koerner SK. Delayed development of obliterative bronchiolitis syndrome with OKT3 after unilateral lung transplantation. A plea for multicenter immunosuppressive trials. Chest. 1996;109:870-3.

54. Diamond DA, Michalski JM, Lynch JP, Trulock EP 3rd. Efficacy of total lymphoid irradiation for chronic allograft rejection following bilateral lung transplantation. Int J Radiat Oncol Biol Phys. 1998;41:795-800.
55. Davis RD Jr, Lau CL, Eubanks S, Messier RH, Hadjiliadis D, Steele MP, Palmer SM. Improved lung allograft function after fundoplication in patients with gastroesophageal reflux disease undergoing lung transplantation. J Thorac Cardiovasc Surg. 2003;125:533-42.
56. Treede H, Klepetko W, Reichenspurner H, Zuckermann A, Meiser B, Birsan T, Wisser W, Reichert B; Munich and Vienna Lung Transplant Group. Tacrolimus versus cyclosporine after lung transplantation: a prospective, open, randomized two-center trial comparing two different immunosuppressive protocols. J Heart Lung Transplant. 2001;20:511-7.
57. Soghikian MV, Valentine VG, Berry GJ, Patel HR, Robbins RC, Theodore J. Impact of ganciclovir prophylaxis on heart-lung and lung transplant recipients. J Heart Lung Transplant. 1996;15:881-7.
58. Otterbein LE, Choi AM. Heme oxygenase: colors of defense against cellular stress. Am J Physiol Lung Cell Mol Physiol. 2000;279:L1029-37
59. Morse D, Choi AM. Heme Oxygenase-1. The "emerging molecule" has arrived. Am J Respir Cell Mol Biol 2002;27:8-16.
60. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat.Med 2000;6:422-428.
61. Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat.Med 1996;2: 87-90.
62. Stefan W Ryter, Leo E Otterbein, Danielle Morse, Augustine MK Choi. Heme oxygenase/ carbon monoxide signaling pathways: Regulation and functional significance. Molecular and Cellular Biochemistry 2002;234/235:249 –263.
63. Lu F, Zander DS, Visner GA. Increased expression of heme oxygenase-1 in human lung transplantation. J Heart Lung Transplant 2002;21:1120-126.
64. Ke B, Buelow R, Shen XD, Melinek J, Amersi F, Gao F, Ritter T, Volk HD, Busuttil RW, Kupiec-Weglinski JW. Heme Oxygenase 1 Gene Transfer Prevents CD95/Fas Ligand-Mediated Apoptosis and Improves Liver Allograft Survival via Carbon Monoxide Signaling Pathway. Hum Gene Ther 2002;13: 1189-99.
65. Tobiasch E, Gunther L, Bach FH. Heme oxygenase-1 protects pancreatic beta cells from apoptosis caused by various stimuli. J Investig Med 2001;49:566-71.
66. Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, Grey ST, Colvin RB, Choi AM, Poss KD, Bach FH. Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat.Med 1998; 4:1073-1077.
67. Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. J Immunol. 2001;166:4185-94.
68. Soncul H, Oz E, Kalaycioglu S. Role of ischemic preconditioning on ischemia-reperfusion injury of the lung. Chest 1999;115:1672-7.
69. Katori M, Buelow R, Ke B, Ma J, Coito AJ, Iyer S, Southard D, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an antiapoptotic pathway. Transplantation 2002;73: 287-92.
70. Zhang X, Shan P, Otterbein LE, Alam J, Flavell RA, Davis RJ, Choi AM, Lee PJ. Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. J Biol Chem 2003;278:1248-58.
71. Cooper, J. D., M. E. Billingham, T. Egan, M. I. Hertz, T. L. Higenbottam, J. Lynch, J. Mauer, I. Paradis, G. A. Patterson, C. Smith, E. P. Trulock, C. Vreim, and S. Yousem. A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation. J Heart Lung Transplant. 1993;12:713-716
72. Haslam PL, Baughman RP. Report of ERS Task Force: guidelines for standardization of BAL. ERJ. 1999(14):245-8.

Part I.

Assessment of airway inflammation in bronchiolitis obliterans after lung transplantation





Longitudinal profile of bronchoalveolar lavage cell characteristics in patients with a good outcome after lung transplantation

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Abstract

Bronchoalveolar lavage fluid (BALF) analysis is used in patients after lung transplantation (LTX) to obtain more insight into pathological conditions such as acute and chronic allograft rejection. Information on the normal course of BALF cell characteristics in patients with "good outcome" after LTX is limited. Therefore we analyzed 169 BALF samples from 63 well-defined "good outcome" patients after LTX (no acute or chronic transplant dysfunction, bacterial, fungal, or viral infections at the time of BAL). Total cell count decreased from the first months: median (range) 234 x 10^3 (70-610) cells/ml to 103 x 10^3 (10-840) cells/ml during the second year posttransplantation ($p < 0.001$). Cell differential counts did not change during the 2-yr study period. The CD4/CD8 ratio increased significantly from 0.32 (0.11-0.46) just posttransplantation to 0.62 (0.16-4.27) the second year after LTX. This increasing ratio was mainly due to a sharp decreasing $CD8^+$ cell count. Thus, characteristics of BAL cellular patterns in patients with good outcomes after LTX show important changes over time.
We have defined control values for the BALF cellular profile in patients without pathological airway conditions after LTX. We propose to use these control values as a tool for diagnosing patients with pulmonary complications after LTX and for the follow-up of treatment regimens.

INTRODUCTION

Lung transplantation (LTX) is the ultimate and often last therapeutic option for several end-stage lung diseases. The outcome after LTX is mainly determined by the development of chronic graft failure: obliterative bronchiolitis (OB). The latter may even develop during the first months posttransplantation and is the main cause of death after the first 6 mo posttransplantation. Furthermore, OB strongly limits the personally gained quality of life by LTX (1, 2). Early identification of patients "at risk" for development of OB is very important, because intervention by modulating the immunosuppressive regimens may prevent or slow down the "natural course" of the developing chronic graft failure. In contrast, once OB has developed only retransplantation is therapeutically available (3).

Besides transbronchial biopsies and lung function testing, monitoring patients after LTX by bronchoalveolar lavage fluid (BALF) analysis is increasingly used to obtain more insight into the pathogenesis of chronic allograft dysfunction and to diagnose OB (4). Recently we have shown that the differential cell count and cytokine profile of BALF can identify patients "at risk" for developing OB without having abnormal lung function or histopathological findings at that time (6).

To define an abnormal BALF profile, information on the "normal" BALF profile found in patients without further pathological airway conditions after LTX is needed. BALF results from patients after LTX are incomparable to BALF results from healthy subjects possibly due to the immunological allograft response and the immunosuppressive therapeutic regimens (9). Furthermore, BALF profiles are likely to undergo a dynamic change the first years after LTX (12). When reviewing the literature on BALF analysis in "good outcome" LTX groups, a large variability between studies is found with respect to the procedures used for BALF sampling, timing of the BAL procedure (days after LTX), and quantified cellular characteristics of BALF. This indicates the difficulty in defining "normal values," even though all patients in the reviewed studies are being described as "healthy" or "having no complications" (Table 1) (4, 5, 8, 10, 11, 16).

The aim of the present study was to define the "normal" cellular profile of BALF of patients after LTX without any signs or symptoms of accompanying airway pathology. Therefore, in a prospective cohort of LTX patients, we set out to assess BALF characteristics in a well-defined subpopulation of "good outcome" LTX patients in whom BAL was performed at fixed intervals during the first 2 yr after LTX.

METHODS

Patients and Study Design

A prospective cohort study analysis was performed on BALF samples from all patients transplanted in our center between November 1990 and December 1998. BALF samples were excluded for analysis when acute rejection; OB (histological and/or functional decline within 2 yr after the last BAL); bacterial respiratory tract infections (also clinical diagnosis of purulent bronchitis made by bronchoscopy); or fungal, cytomegalovirus (CMV), or other viral infections were present at the time of the BAL procedure. Single, bilateral, and heart-lung TX were performed according to established techniques (22).

Diagnostic Protocol and Follow-up

Graft function was assessed by pulmonary function testing and routinely performed transbronchial biopsies before discharge and every 6 mo after LTX (6). Acute allograft rejection was diagnosed clinically or histopathologically as defined by Yousem and coworkers (23). Diagnosis of OB was

TABLE 1. BRONCHOALVEOLAR LAVAGE FLUID PROFILES IN PATIENTS WITH GOOD OUTCOME AFTER LTX: REVIEW OF THE LITERATURE*

Author (yr)	n† (pts)	FEV_1 (%pred)	Time post-TX (d)	Method‡	Recovery (%)	Total Cell (× 10^3/ml)	AM (%)	LYM (%)	PMN (%)	EO (%)	CD4/CD8
Clelland (16) (1993)	8	—	—	2 × 50 sal		140	88	10	4	—	—
Magnan (17) (1994)	29 (70)	Normal	—	5 × 50 sal	—	286 (± 40.6)	73 (± 19)	12.4 (± 1.3)	11 (± 2.3)	—	—
Whitehead (18) (1995)	14	—	—	3 × 60 pbs	—	—	86.7 (± 7.8)	8.9 (± 6.5)	4.0 (± 2.0)	—	—
Magnan (19) (1996)	6 (23)	Normal	—	5 × 50 sal	—	442 (± 108)	75.6 (± 2.1)	10.4 (± 1.5)	11.7 (± 3.1)	—	—
DiGiovine (4) (1996)	41	—	518 (± 83)	4 × 60 sal	—	—	89.1	4.6	5 8	0.6	—
Riise (20) (1997)	? (58)	—	—	2 × 80 pbs	54 (± 11)	300 (± 200)	79 (± 20)	6 (± 6)	12 (± 19)	0.2 (± 0.5)	—
Ward (10) (1998)	17	98.6 (± 2.9)	190 (58–1,301)	3 × 60 pbs	50 (22–62)	280 (100–1,700)	71 (20–91)	22 (3–78)	4 (0.4–18)	0 (0–3)	—
Riise (21) (1998)	13	—	732 (274–3,300)	2 × 60 pbs	—	—	74 (55–96)	22 (0.5–42)	3.8 (0.5–7)	0 (0–1)	—
Riise (5) (1999)	20	—	—	2 × 80 pbs	50 (48–64)§	—	85.5 (76–91)§	7.0 (4.1–13.1)§	5.5 (3.0–9.0)§	0.0 0.0	—
Elssner (11) (2000)	9	100.7 (± 1.6)	485 (± 77)	5 × 20 sal	36 (± 5.8)	139 (± 44)	94.8 (± 2.5)	1.2 (± 1.1)	4.1 (± 1.3)	0.3 (± 0.2)	—
Zheng (8) (2000)	19	98.9 (± 1.9)	555 (58–1,558)	3 × 60 pbs	47 (± 6.1)	160 (± 50)	73 (± 8)	19 (± 9)	4.7 (± 2.2)	0.3 (± 0.2)	—

Definition of abbreviations: AM = alveolar macrophages; EO = eosinophil; LTX = lung transplantation; LY = lymphocytes; pbs = phosphate-buffered saline; PMN = neutrophils; sal = normal saline.

* Values given as median (min–max) or mean (± SD).

† n = number of patients, with the number of BAL samples in parentheses.

‡ Method of BALF sampling.

§ Interquartile range.

Figure 1. Longitudinal profile of the total cell count (alveolar fraction) of 19 patients with good outcome after LTX.

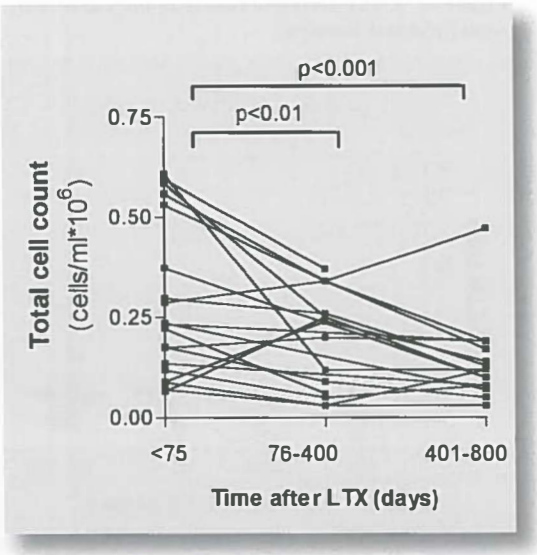


Figure 2. Cross-sectional course of the total cell count of the alveolar fraction of 63 patients with good outcome after LTX.

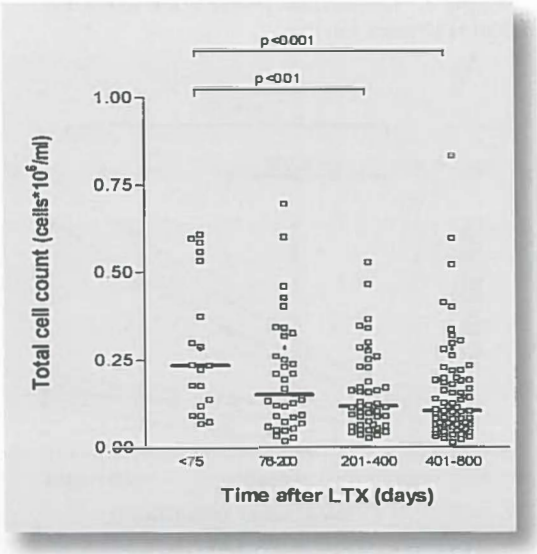


TABLE 2. RECOVERY AND CELL COUNTS OF BALF IN PATIENTS WITH GOOD OUTCOME AFTER LTX*

	Days after LTX							
	< 75		76–200		201–400		400–800	
	BF	AF	BF	AF	BF	AF	BF	AF
Number of BALF	17	19	27	34	45	47	50	69
Recovery, %	25 (3–55)	71 (31–86)	25 (5–85)	69 (28–83)	25 (8–73)	69 (2–87)	25 (1–100)	63 (19–85)
Total cell, × 10^3/ml	173 (10–960)	234 (70–610)	100 (10–820)	151 (20–700)	100 (0–510)	116 (30–530)†	90 (0–830)	103 (10–840)‡
AM, %	92 (39–97)	93 (76–98)	94 (23–98)	93 (76–99)	92 (61–100)	94 (70–99)	92 (60–100)	96 (72–100)
PMN, %	2.5 (1–57)	2 (0–13)	2 (0–73)	2 (0–10)	2 (0–34)	2 (0–22)	2.9 (0–38)	1 (0–24)
LY, %	2.5 (1–10)	4 (0–23)	2 (0–33)	3 (1–22)	2.5 (0–26)	3 (0–28)	2 (0–12)	2 (0–16)†
EO, %	0 (0–1)	0.18 (0–1)	0 (0–1)	0.16 (0–1)	0 (0–4)	0.18 (0–5)	0 (0–3)	0.14 (0–5)

Definition of abbreviations: AF = alveolar fraction; AM = alveolar macrophages; BALF = bronchoalveolar lavage fluid; BF = bronchial fraction; EO = eosinophils; LTX = lung transplantation; LY = lymphocytes; PMN = neutrophils.

* Values are presented as medians (range). n = number of BALF samples analyzed. Values given cross-sectional in four consecutive time periods after LTX.

† $p < 0.01$, ‡ $p < 0.001$ when comparing the different time periods with < 75 d after LTX.

TABLE 3. BALF LONGITUDINAL CHARACTERISTICS OF TOTAL CELL COUNT, CELL DIFFERENTIATION, $CD3^+$, AND CD4/CDB RATIO IN PATIENTS WITH GOOD OUTCOME AFTER LTX

Time after LTX, d	< 75	76–400	401–800
Number of BALF samples	19	15	16
Total cell count, × 10^3/ml	234 (70–610)	240 (30–370)*	120 (30–470)†
Macrophages, %	93 (76–98)	91 (76–99)	97 (90–98)
Lymphocytes, %	5 (0–23)	3 (1–22)	1.5 (1–7)‡
Neutrophils, %	2 (0–13)	3 (0–16)	1.5 (0–4)
Eosinophils, %	0 (0–1)	0 (0–0)	0 (0–1)
Number of FACS samples	12	11	10
$CD3^+$, %	90 (81–98)	85.5 (59–95)	80.5 (64–97)†
CD4/CD8 ratio	0.32 (0.11–0.63)	0.49 (0.15–1.52)‡	0.74 (0.21–4.27)*

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; FACS = fluorescence-activated cell sorter; LTX = lung transplantation.

* $p < 0.01$, † $p < 0.001$, ‡ $p < 0.05$ when comparing the different time periods with < 75 d after LTX.

Figure 3. Longitudinal profile of the CD4/CD8 ratio (alveolar fraction).

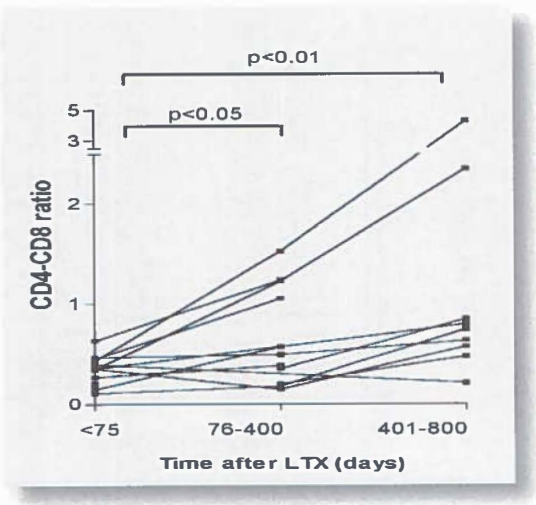
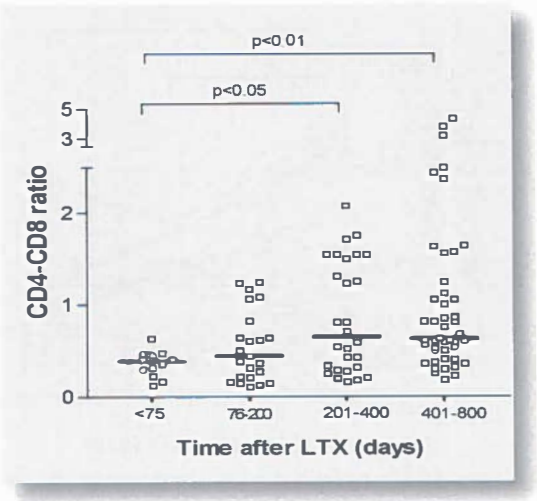


Figure 4. Cross-sectional course of the CD4/CD8 ratio (alveolar fraction).



based on a classification and grading by the ISHLT (23). Diagnosis of bacterial or fungal infections was based on positive BALF cultures. Active CMV infection was assessed by CMV serology and CMV antigenemia testing as described before (6, 24, 25).

Therapeutic Protocol

Immunosuppression included up to five gifts of antithymocyte globulins (rATG, Merieux, dosed at 3 mg/kg) in the first 10 d after transplantation. The maintenance immunosuppressive regimen consisted of cyclosporin (aimed at serum levels of 400 ng/ml post-LTX, tapered down in 3 wk to 150 ng/ml and maintained at this level for all time periods), azathioprine (1-3 mg/kg/d), and prednisolone (0.1-0.2 mg/kg/d). The newer immunosuppressive drugs tacrolimus and mycophenolate mofetil were not used in the first 7 yr of our study. All patients received acyclovir and cotrimoxazole prophylaxis (6).

Bronchoalveolar Lavage and Cell Isolation

BAL and bronchoscopy were routinely performed after the first month and every 6 mo after LTX and additionally in case of clinical indication. This protocol was approved by the Medical Ethics Committee. Two aliquots of 20 ml and three aliquots of 50 ml prewarmed phosphate-buffered saline (PBS) were instilled. The first 20 ml portion was investigated for viruses, bacteria, and fungi, the second 20 ml portion was isolated as bronchial fraction (BF), and the three 50 ml fractions were pooled for the alveolar fraction (AF) (6, 26, 27). The BF and AF were further processed for leukocyte differentiation as described earlier (6) and FACS. The differentiation was determined by counting 200 cells on two slides each.

FACS Analysis

To quantify percentage and total number of lymphocyte subtypes direct immunofluorescence in the AF was performed with monoclonal antibodies (MAb) phycoerythrin and fluorescein isothiocyanate conjugated. The following MAbs were used: CD3/CD4, CD3/CD8 (IQ-Products), CD4/CD25, HLADR/CD4, HLADR/CD8 (Beckton Dickinson), and CD45RO/CD4, CD45RO/CD8 (Dako, Denmark). Briefly, one million cells of the AF were incubated for 30 min at 4° C with 10 μl of MAb followed by centrifugation at 590 x g for 5 min at 10° C and washing two times with 0.5% (wt/vol) bovine serum albumin (BSA) in PBS. Labeled cells were analyzed using a fluorescence-activated cell sorter-Calibur (Beckton Dickinson).

Statistical Analysis

Data were analyzed using SPSS/PC$^+$ software (SPSS Benelux b.v., Gorinchem, The Netherlands). Cell counts and lymphocyte subtypes were compared using the nonparametric Wilcoxon signed rank test for longitudinal analysis. The differences shown in the cross-sectional data are calculated on longitudinal analysis. p Values < 0.05 were considered as significant. All results are presented as median and range, unless otherwise stated.

RESULTS

Bronchoalveolar Lavage Selection

BALF samples (501) were collected between 1990 and 1998. A total of 201 samples were excluded for further analysis because of the death of the respective individual or development of OB within 2 yr of the last BAL. Of the remaining 300 samples, 67 were excluded because of signs or symptoms of infectious complications (bacterial, viral, or fungal) and 64 samples were excluded thereafter because of acute rejection at time of BAL. The remaining 169 BALF samples were included for analysis. The BALF samples were distributed over four time periods (< 75, 76-200, 201-400, and 401-800 d after LTX) and cross-sectionally analyzed

for cellular characteristics (Table 2). The BALF samples included in the first time period after LTX (< 75 d) were also longitudinally analyzed (Table 3). The longitudinal samples were distributed over three time periods (< 75, 76-400, and 401-800 d after LTX). Of these 169 BALF samples, all alveolar fractions (AF) and 139 bronchial fractions (BF) were adequate to analyze. In 30 BF samples, no recovery of the instilled fluid was obtained.

Patient Characteristics

The 169 BALF samples were obtained from 63 patients (32 female, 31 male) with a mean age of 43.4 yr (SD ± 10.4 yr) at time of transplantation. Ten of these patients received single LTX, 1 heart-lung TX, and 52 bilateral LTX. Six patients had lung fibrosis as pretransplant diagnosis, 21 α_1-antitrypsin (α_1-AT) deficiency (all without augmentation treatment), 11 chronic obstructive pulmonary disease (COPD), 5 bronchiectasis, 5 primary pulmonary hypertension, 5 secondary pulmonary hypertension, and 10 cystic fibrosis pre-LTX.

Recovery

Recovery of both the bronchial and alveolar fraction percentages was comparable between all time periods, although the percentage recovered fluid was much larger in the alveolar fraction: 66% (2-87) when compared with the bronchial fraction: 25% (1-100) (Table 2).

Cellular Content and Differentiation

In the longitudinal data analysis, total cell count (AF) decreased from 234 x10^3 (70-610 x10^3) cells/ml in the first BAL within 75 d after LTX to 120 x10^3 (30-470 x10^3) cells/ml during the second year posttransplantation ($p < 0.001$) (Figure 1). Total cell counts had the same profile in the cross-sectional data: a decreasing total cell count from 234 x10^3 (70- 610 x10^3) cells/ml (< 75 days) to 103 x10^3 (10-840 x10^3) cells/ml during the second year post-LTX (Figure 2). Overall, cell differentiation in the alveolar fraction was characterized by a predominance of alveolar macrophages: 94% (70-100) and low numbers of lymphocytes: 2% (0-24) and neutrophils: 3% (0-28). In the longitudinal analysis (Table 3) as well as in the cross-sectional data (Table 2), a slight decrease in lymphocyte percentage was shown: 5% (0-23) < 75 d to 1.5% (1) 401-800 d post-LTX ($p < 0.05$). No diffe-

TABLE 4. CROSS-SECTIONAL LYMPHOCYTE SUBTYPE ANALYSIS (FACS) OF BALF CELLS OF PATIENTS WITH GOOD OUTCOME AFTER LTX*

Time after LTX, d	< 75	76–200	201–400	401–800
N	12	24	29	45
$CD3^+$, %	90 (81–98)	91 (59–95)	87 (65–98)	83 (51–99)†
$CD4^+$, %	21 (9–30)	24 (7–47)	30 (11–62)†	30 (10–79)‡
$CD8^+$, %	65 (46–80)	59 (23–82)†	48 (17–82)‡	44 (15–64)§
$CD4^+$, 10^4/ml	2.62 (0.61–8.62)	2.07 (0.13–8.99)	1.50 (0.14–6.78)	0.87 (0.16–13.3)†
$CD8^+$, 10^4/ml	7.78 (2.19–43.0)	4.71 (0.12–44.20)†	2.33 (0.19–19.5)‡	1.55 (0.17–15.9)§
CD4/CD8	0.32 (0.11–0.46)	0.40 (0.09–1.23)	0.69 (0.15–2.1)†	0.62 (0.16–4.27)‡
$CD4^+$HLADR, %	63 (45–83)	53 (14–93)	50 (27–99)†	52 (18–98)†
$CD8^+$HLADR, %	64 (43–89)	51 (13–99)†	45 (19–99)‡	45 (8–89)‡
$CD4^+CD45^+$, %	100 (94–100)	100 (95–100)	100 (96–100)	99 (89–100)
$CD8^+CD45^+$, %	93 (88–100)	88 (53–100)†	87 (63–100)†	89 (64–100)†
$CD4^+CD25^+$, %	46 (0–68)	21 (3–62)†	16.5 (1–54)†	24 (2–57)‡

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; FACS = fluorescence-activated cell sorter; LTX = lung transplantation.
* Values are presented as medians (range). n = number of BALF samples analyzed.
† $p < 0.05$, ‡ $p < 0.01$, § $p < 0.001$ when comparing the time periods with < 75 d after LTX.

rence was found in the cell differentiation of the sequential BF (Table 2).

Lymphocyte Subtype Analysis (AF Only)

CD3+:

A slight, but significant decrease in total $CD3^+$ cell count and percentage $CD3^+$ lymphocytes was noticed in both the longitudinal (Table 3) and the cross-sectional analysis (Table 4).

$CD4^+/CD8^+$

The CD4/CD8 ratio for the longitudinal follow-up increased significantly from 0.32 (0.11-0.63) just posttransplantation to 0.74 (0.21-4.27) the second year after LTX ($p < 0.01$) (Figure 3). These data are supported by the cross-sectional data in which a rise from 0.32 (0.11-0.46) just posttransplantation to 0.62 (0.16-4.27) the second year after LTX occurred (Figure 4). The increase in the CD4/CD8 ratio was mainly due to a sharp decrease in total $CD8^+$ T cells from 7.78×10^4 cells/ml (2.19-43.0) < 75 d after LTX to 1.55×10^4cells/ml (0.17-15.9) for 401-800 d after LTX ($p < 0.001$), with a corresponding percentage $CD8^+$ cells of 65% (46-80) and 44% (15-64), respectively ($p < 0.001$). The total $CD4^+$ cell count dropped from 2.62×10^4 cells/ml (0.61-8.62) < 75 d post-LTX to 0.87×10^4cells/ml (0.16-13.3) at 401-800 d post-LTX ($p < 0.05$), but the percentage increased from 21% (9) to 30% (10-79) at these same time periods ($p < 0.01$) (Figure 5, Table 4).

Activation markers

Both $CD4^+$HLADR and $CD8^+$HLADR expression decreased in the first months after LTX and remained stable after the first 75 d (Table 4). $CD4^+$CD45Ro remained unaffected after LTX and $CD8^+$CD45Ro showed a slight decrease (Table 4). Furthermore, $CD4^+CD25^+$ T cells showed a significant decrease from 46% (0-68) < 75 d after LTX to 21%, 16.5%, and 24% at the subsequent periods after LTX ($p < 0.01$) (Table 4).

Subgroup Analysis of the α_1-AT-Deficient Recipients

Because a large number ($n = 21$) of the included patients are α_1-AT deficient (a subgroup that is showing a lower incidence of OB [1]) we performed the same analysis on these patients. The results were the same as in the whole group. Total cell counts decreased from 235×10^3 (range 320) in the first time period (< 75 d after LTX) to 100×10^3 (620) in the last time period (400-800 d after LTX), $CD3^+$ from 87% (12) to 83% (43), $CD4^+$ from 21% (15) to 30% (50), $CD8^+$ from 67% (17) to 37% (43), $CD4^+CD25^+$ from 49.5% (7) to 19% (41), and finally the CD4/CD8 ratio from 0.41 (0.23) to 0.80 (4.11).

This study is the first to assess the normal longitudinal profile of cellular BALF characteristics after LTX. These data were determined in patients who can be considered as "normals," thus without any complications or signs or symptoms of additional airway pathology after LTX. We show that there is an important physiological posttransplantation change of the cellular BALF profile. Total cell counts, $CD3^+$, $CD4^+$, $CD8^+$, and $CD4^+/CD25^+$ lymphocyte counts and CD4/CD8 ratio all undergo a dynamic change during the first 2 yr after LTX.

DISCUSSION

Cellular and immunological contents of the bronchial fraction of BALF have been shown to discriminate patients at risk for developing OB very early after LTX. Therefore, we analyzed both the bronchial fraction, which is assumed to represent the proximal airways, and the alveolar fraction, reflecting peripheral airways (6, 26). Cell differential counts were found to be comparable in both fractions in our "good outcome" patients (Table 2). Thus, there is no advantage of BF over AF in this respect or vice versa.

Previous studies have published data on

cellular and immunological characteristics of BALF in "good outcome" LTX groups in relation to the pathogenesis and diagnosis of OB and acute rejection episodes (Table 1) (4, 5, 8, 10, 11, 16). However, our review of these studies shows that there are large differences in the total and differential cell counts between these studies, even though the "good outcome" groups were always defined as stable, without infection and rejection. Possible explanations for these discrepant findings may be found in the difference in timing in performing the BAL procedure after LTX, as well as different immunosuppressive regimens and laboratory protocols for BALF analysis used (Table 1). It has been suggested that the total cell count remains high for years. A high total cell count has indeed been described by several authors the first weeks post-LTX only, as has been confirmed by our results (9, 12). Our study now shows that when a "good outcome" group is defined, there is

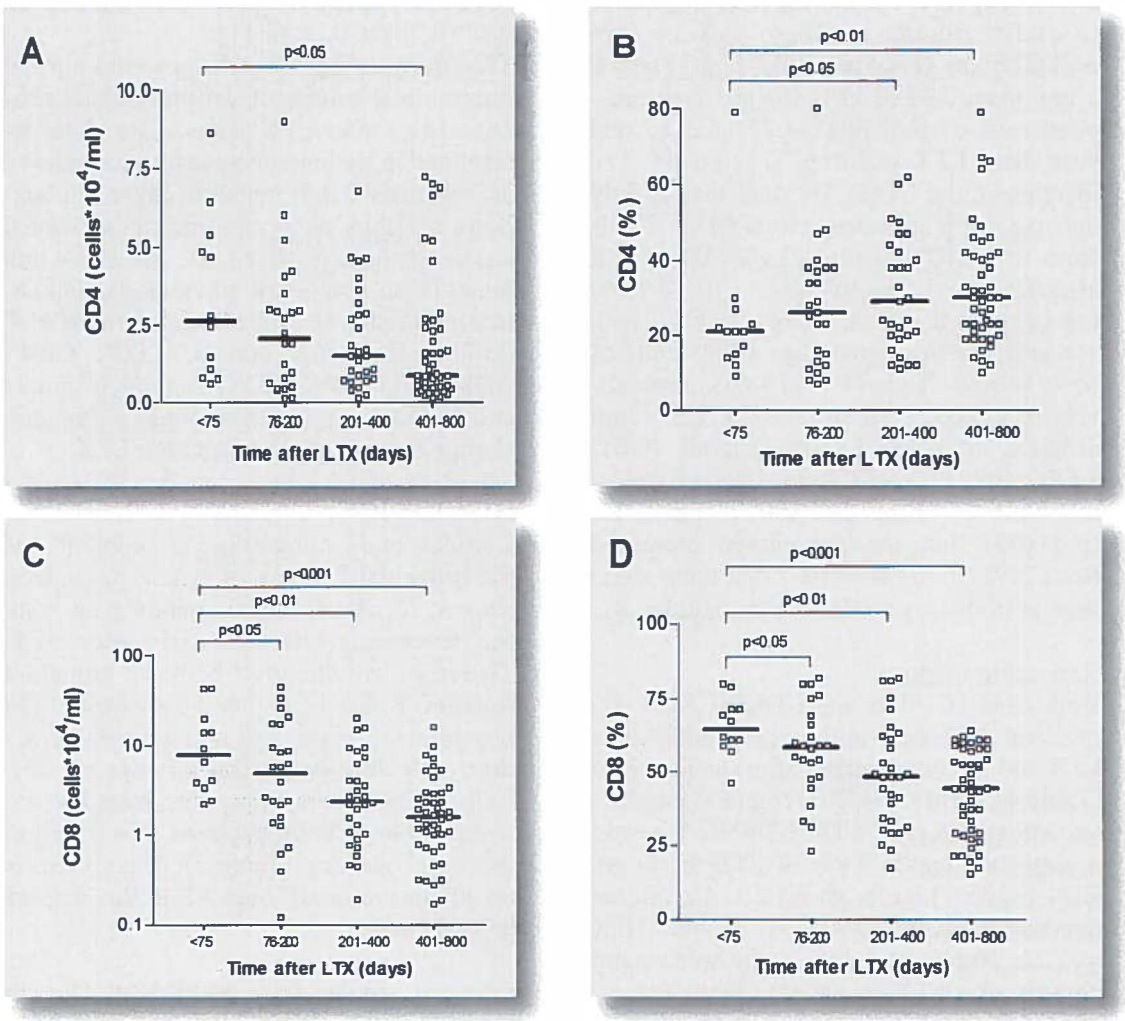


FIGURE 5. *Cross-sectional absolute (A, C) and percentage (B, D) cell counts for CD4⁺ and CD8⁺ cells showing the much more pronounced decrease in CD8⁺ cells after LTX.*

a definite decline in total cell count 1 yr post-LTX.

We analyzed 169 BALF samples (Figure 1) from 63 patients after LTX in whom no signs of OB occurred within 2 yr after the last BAL and no signs of acute rejection; acute bronchitis; or bacterial, fungal, or viral infections were present at the time of the BAL procedure. Thus, without reasonable doubt, we selected and investigated the BALF samples that can provide as clearly as possible insight in the normal course of alveolar cell influx in LTX patients. We therefore propose using these data for further evaluation of BALF cellular profiles in individuals post-LTX.

One may wonder whether the BALF cellular characteristics in "good outcome" patients after LTX are in the normal range, thus comparable to BALF cellular characteristics in healthy nonsmoking subjects. When comparing the BALF cellular profile in healthy nonsmoking subjects previously studied in our laboratory with our "good outcome" patients 401-800 d after LTX, it appears that cell counts in this LTX group are reasonably within the normal range: total cell counts LTX: median 103 $x10^3$/ml (range 10-840) versus healthy 100-150 $x10^3$/ml; neutrophil counts LTX: 1% (0-24) versus healthy 0-2%. The percentage alveolar macrophages seems to be higher in our subjects (85-92% in healthy subjects) and the lymphocyte percentage is lower compared with normal subjects (7-12% in healthy subjects) (27, 28, 29). Marked differences are found in the lymphocyte subtypes in BALF between our "good outcome" LTX subjects (401-800 d) when compared with reference values for nontransplanted, nonsmoking, healthy subjects. First, the percentage T cells ($CD3^+$) is lower in the healthy subjects (50-73%) when compared with our LTX population (median 83%, range 51-99). Furthermore, there is a much lower CD4/CD8 ratio in the LTX group: 0.62 (0.16-4.27) in our LTX group the second year after transplantation versus 1.5-2.0 in normal subjects. Two years after LTX, the total $CD4^+$ count of the "good outcome" LTX patients reaches the normal range for healthy persons, whereas the total $CD8^+$ count remains high above the normal values: 1.55 $x10^4$ cells/ml (LTX) versus 0.5 $x10^4$ cells/ml (healthy persons) (28, 29). This high percentage $CD8^+$ may indicate ongoing allograft response. Our findings are also supported by Farver and coworkers who recently showed a high percentage of $CD8^+$ T cells in subjects with LTX rejection and nonrejection when compared with nonsmoking healthy subjects. The corresponding percentage $CD4^+$ cells was lower for the LTX patients when compared with the healthy subjects (30).

A longitudinal change in the BALF cellular profile is to be expected after LTX because of the abrupt start of allograft response, with responding humoral and cellular host defense, the initiated aggressive immunosuppressive therapy, and the knowledge that approximately 3 mo after LTX the donor cells are replaced by recipient cells (9). A longitudinal course has also been suggested by two earlier studies performing serial BALF analysis after LTX in unselected patients (12, 13). Defining the normal course in BALF cellular characteristics after LTX in a longitudinal set-up has not been determined so far in a properly selected group of patients post-LTX without any other pulmonary complications. Our results show a constantly increasing CD4/CD8 ratio over the first 2 yr post-LTX. This increasing CD4/CD8 ratio is due to a mild decline in total $CD4^+$ cell count and a much more pronounced decline of the total $CD8^+$ cell count, thus providing a rising percentage $CD4^+$ and decreasing percentage $CD8^+$ lymphocytes. These results are not compatible with current available literature

on sequentially performed analysis of CD4/CD8 ratios the first year after LTX. The three published studies (with 14 patients evaluated in total) suggest a post-LTX decrease in CD4/CD8 ratio (13, 14, 18). In contrast, in our well-defined population, we observed an increasing percentage in $CD4^+$ cells resulting in a rising CD4/CD8 ratio. This observation is supported by the study of Crim and coworkers, who also noticed a rise in the percentage $CD4^+$ cells and CD4/CD8 ratio after LTX in 10 "good outcome" patients (15).

The dynamic change in, for example, $CD8^+$ counts after LTX has important bearings on the outcome of previously performed studies using flow cytometric data, as is illustrated by the studies performed by Ward and Snell (10, 31). They found no difference in flow cytometric data between OB and stable LTX patients, yet with a large difference in the time post-LTX at which the BAL procedures were performed: 855 ± 212 d and 340 ± 323 d, respectively, that is, in our study $CD8^+$ counts declined for this difference in time from 4.71×10^4 cells/ml to 1.55×10^4 cells/ml. If Ward and Snell had used "time post-LTX" matched groups, they probably would have found a difference in the flow cytometric data between OB and their stable LTX patients (10, 31).

Our findings of a dynamic change in $CD4^+$ and $CD8^+$ cell count and thus the CD4/CD8 ratio after LTX can be explained by the direct and ongoing allograft response and the immunosuppressive treatment given. The direct allograft response ensures a high total $CD8^+$ cell count the first weeks after transplantation, and this decreases due to the strong immunosuppressive blockage of cyclosporin or tacrolimus on the $CD4^+$ (Th1) cells. The graft recipient $CD4^+$ cells are directly activated due to direct recognition of cell surface markers of donor APCs. The $CD4^+$ cells are preferentially activated into Th1 cells, which induce a delayed type hypersensitivity reaction resulting in acute allograft rejection (32, 33).

We also investigated lymphocyte activation markers. We found a high percentage of $CD4^+CD25^+$ lymphocytes (α–chain of the interleukin-2 receptor) just post-LTX with a decreasing percentage over the following 2 yr, both resembling the results found by Crim and coworkers (15). The high percentage just post-LTX indicates very aggressive reactivity to donor cells in the early postoperative phase. The remaining higher level of $CD4^+CD25^+$ cells when compared with healthy non-LTX subjects probably reflects the ongoing allograft response (10, 15, 32). The number of $CD4^+$HLADR and $CD8^+$HLADR cells started at a highly activated level, thereafter decreasing and leveling off during the first year after LTX to remain stable.
Our findings 2 yr after LTX are comparable to HLA-DR findings of previously reported flow cytometric data (10). When comparing these data with HLA-DR BALF values of healthy non-LTX volunteers, the HLA-DR expression remains at a much higher level for LTX patients, most likely due to the chronic allograft response (32).

From our study we conclude that a decrease in total cell count, lymphocytes, and $CD3^+$ lymphocytes and an increase in the CD4/CD8 ratio after LTX represent the natural course of cells in BALF in patients without pathological airway conditions after LTX. We were able to define control values for the BALF cellular profile in patients with no complications after LTX. We suggest using these control values as a tool for diagnosing patients with pulmonary complications after LTX and for the follow-up treatment regimens. It appears that dynamic post-LTX profiles have to be taken into account when research is performed or

clinical interpretations are made on BALF cellular characteristics after LTX.

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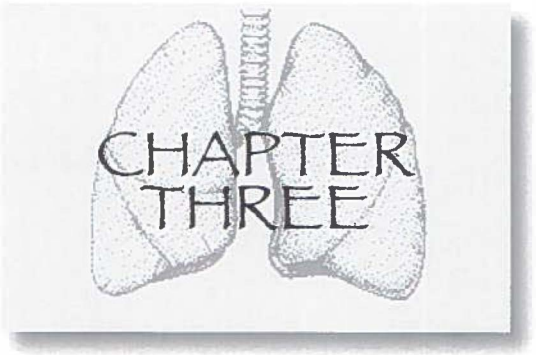
REFERENCES

1. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The registry of the international society for heart and lung transplantation: seventeenth official report. J Heart Lung Transplant 2000; 19: 909-931
2. Van den Berg JWK, Geertsma A, van der Bij W, Koëter GH, de Boer WJ, Postma DS, ten Vergert EM. Bronchiolitis obliterans syndrome after lung transplantation and health-related quality of life. Am J Respir Crit Care Med 2000; 161: 1937-1941
3. Revell MP, Lewis ME, Llewellyn-Jones CG, Wilson IC, Bonser RS. Conservation of small-airway function by tacrolimus/cyclosporine conversion in the management of bronchiolitis obliterans following lung transplantation. J Heart Lung Transplant 2000; 19: 1219-1223
4. DiGiovine B, Lynch JP III,, Martinez FJ, Flint A, Whyte RI, Iannettoni MD, Arenberg DA, Burdick MD, Glass MC, Wilke CA, Morris SB, Kunkel SL, Strieter RM. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. J Immunol 1996; 157: 4194-4202
5. Riise GC, Andersson BA, Kjellstrom C, Martensson G, Nilsson FN, Ryd W, Schersten H. Persistent high BAL fluid granulocyte activation marker levels as early indicators of bronchiolitis obliterans after lung transplant. Eur Respir J 1999; 14: 1123-1130
6. Scholma J, Slebos DJ, Boezen HM, van den Berg JWK, van der Bij W, de Boer W, Koëter GH, Timens W, Kauffman HF, Postma DS. Eosinophilic granulocytes and interleukin-6 level in bronchoalveolar lavage fluid are associated with the development of obliterative bronchiolitis after lung transplantation. Am J Respir Crit Care Med 2000; 162: 2221-2225
7. Henke JA, Golden JA, Yelin EH, Keith FM, Blanc PD. Persistent increase of BAL neutrophils as a predictor of mortality following lung transplant. Chest 1999; 115: 403-409
8. Zheng L, Walters EH, Ward C, Wang N, Orsida B, Whitford H, Williams TJ, Kotsimbos T, Snell GI. Airway neutrophilia in stable and bronchiolitis obliterans syndrome patients following lung transplantation. Thorax 2000; 55: 53-59
9. Tiroke AH, Bewig B, Haverich A. Bronchoalveolar lavage in lung transplantation. State of the art. Clin Transplant 1999; 13: 131-157
10. Ward C, Snell GI, Zheng L, Orsida B, Whitford H, Williams TJ, Walters EH. Endobronchial biopsy and bronchoalveolar lavage in stable lung transplant recipients and chronic rejection. Am J Respir Crit Care Med 1998; 158: 84-91
11. Elssner A, Jaumann F, Dobmann S, Behr J, Schwaiblmair M, Reichenspurner H, Furst H, Briegel J, Vogelmeier C. Elevated levels of interleukin-8 and transforming growth factor-beta in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells. Transplantation 2000; 70: 362-367
12. Prior CP, Klima G, Gattringer C, Schimdt T, Margreiter R. Serial bronchoalveolar lavage in heart-lung transplant recipients: cytological and microbiologic findings. Transplant Proc 1989; 21: 2588-2589
13. Maurer JR, Gough E, Chamberlain DW, Patterson GA, Grossman RF. Sequential bronchoalveolar lavage studies from patients undergoing double lung and heart-lung transplant. Transplant Proc 1989; 21: 2585-2587
14. Holland V, Windsor N, Brousseau K, Lloyd K, Noon G, Whisennand H, DeBakey M, Lawrence E. Serial changes in bronchoalveolar lavage following heart-lung and single lung transplantation. Am Rev Respir Dis 1989;A240.
15. Crim C, Keller C, Dunphy C, Maluf H, Ohar J. Flow cytometric analysis of lung lymphocytes in lung transplant recipients. Am J Respir Crit Care Med 1995; 153: 1041-1046 .
16. Clelland C, Higenbottam T, Stewart S, Otulana B, Wreghitt T, Gray J, Scott J, Wallwork J. Bronchoalveolar lavage and transbronchial biopsy during acute rejection and infection in heart-lung transplant patients. Am Rev Respir Dis 1993; 147: 1386-1392
17. Magnan A, Mege JL, Reynaud M, Thomas P, Capo C, Garbe L, Meric B, Badier M, Bongrand P, Viard L. Monitoring of alveolar macrophage production of tumor necrosis factor-alpha and interleukin-6

in lung transplant recipients Am J Respir Crit Care Med 1994; 150: 684-689
18. Whitehead BF, Stoehr C, Finkle C, Patterson G, Theodore J, Clayberger C, Starnes VA. Analysis of bronchoalveolar lavage from human lung transplant recipients by flow cytometry. Respir Med 1995; 89: 27-34
19. Magnan A, Mege JL, Escallier JC, Brisse J, Capo C, Reynaud M, Thomas P, Meric B, Garbe L, Badier M, Viard L, Bongrand P, Giudicelli R, Metras D, Fuentes P, Vervloet D, Noirclerc M. Balance between alveolar macrophage IL-6 and TGF-beta in lung-transplant recipients. Am J Respir Crit Care Med 1996; 153: 1431-1436
20. Riise GC, Kjellstrom C, Ryd W, Schersten H, Nilsson FN, Martensson G, Andersson BA. Inflammatory cells and activation markers in BAL during acute rejection and infection in lung transplant recipients: a prospective, longitudinal study. Eur Respir J 1997; 10: 1742-1746
21. Riise GC, Williams A, Kjellstrom C, Schersten H, Andersson BA, Kelly FJ. Bronchiolitis obliterans syndrome in lung transplant recipients is associated with increased neutrophil activity and decreased anti-oxidant status in the lung. Eur Respir J 1998; 12: 82-88
22. Cooper JD. The evolution of techniques and indications for lung transplantation. Ann Surg 1990; 212: 249-255
23. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Tazelaar HD. Revision of the 1990 Working Formulation for the Classification of Pulmonary Allograft Rejection: Lung Rejection Study Group. J Heart Lung Transplant 1996; 15: 1-15
24. Van der Giessen M, van den Berg AP, van der Bij W, Postma DS, van Son WJ, The TH. Quantitative measurement of CMV-specific IgG- and IgM-antibodies in relation to CMV antigenemia and disease activity in kidney recipients with an active CMV infection. Clin Exp Immunol 1990; 80: 56-61
25. Van der Bij W, Torensma R, van Son WJ, Anema J, Schirm J, Tegzess AM, The TH. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leucocytes. J Med Virol 1998; 25: 179-188 .
26. Kelly CA, Kotre CJ, Ward C, Hendrick DJ, Walthers EH. Anatomical distribution of bronchoalveolar lavage fluid as assessed by digital subtraction radiography. Thorax 1987; 42: 624-628
27. Rutgers SR, Timens W, Kauffman HF, van der Mark THW, Koëter GH, Postma DS. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. Eur Respir J 2000; 15: 109-115
28. Russi TJ, Crystal RG. Use of bronchoalveolar lavage and airway brushing to investigate the human lung. In: Crystal RG, West JB, editors. The lung, 2nd ed. Philadelphia: Raven Publishers; 1997.
29. Harbeck RJ. Immunophenotyping of bronchoalveolar lavage lymphocytes. Clin Diagn Lab Immunol 1998; 5: 271-277
30. Farver CF, Raychaudhuri B, Malur A, Drazba J, Maurer J, Tubbs R, Metha AC, Schilz R, Thomassen MJ. Increased alveolar macrophage nuclear factor-kB activation and macrophage inhibitory protein-1alpha levels in lung transplant patients. Transplantation 2000; 70: 1599-1603
31. Snell G, Ward C, Wilson J, Orsida B, Williams T, Walters E. Immunopathological changes in the airways of stable lung transplant recipients. Thorax 1997; 52: 322-328
32. Shirwan H. Chronic allograft rejection. Do the Th2 cells preferentially induced by indirect alloantigen recognition play a dominant role? Transplantation 1999;27;68:715-726.
33. Holzknecht ZE, Platt JL. The fine cytokine line between graft acceptance and rejection. Nat Med 2000; 6: 497-498
34. Meyer KC, Soergel P. Variation of bronchoalveolar lymphocyte phenotypes with age in the physiologically normal lung. Thorax 1999; 54: 697-700



Cellular, soluble and flowcytometric characteristics of bronchoalveolar lavage fluid in patients with acute and chronic allograft rejection compared with 'good outcome' after lung transplantation

J Heart Lung Transplant, In press

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Abstract

To assess the value of bronchoalveolar lavage fluid in acute and chronic rejection after lung transplantation we analysed bronchoalveolar lavage fluid cellular differential characteristics, lymphocyte subtypes, interleukin-6 and interleukin-8 cytokine levels in patients with exclusively either acute rejection (n=37) or bronchiolitis obliterans (n=48). Both groups were compared with a control group of lung transplantation patients without rejection or infection, matched for the time the lavage was performed after lung transplantation.

The bronchiolitis obliterans group showed a marked neutrophilia, high interleukin-8 and higher $CD4^+CD25^+$ and $CD8^+CD45^+$ bronchoalveolar lavage fluid levels when compared to their stable controls. When using a cut-off point of >3% neutrophils in the lavage, the sensitivity for BO is 87.0%, the specificity 77.6%. The sensitivity of IL-8 for BO when using a cut-off point of >71.4 pg/mL is 74.5%, the specificity 83.3%.

Bronchoalveolar lavage fluid in acute rejection was characterised by a marked lymphocytosis, but showed no difference with stable controls in any of the lymphocyte subtypes studied. When using a cut-off point of ≤ 1% lymphocytes in the lavage, the sensitivity for AR is 40.4%, the specificity 95.6%.

The marked neutrophilia, high interleukin-8 cytokine level and more activated lymphocyte population in bronchiolitis obliterans may indicate ongoing local allograft rejection. In the present study we were not able to show any difference in lymphocyte subtypes between acute rejection and controls.

Cellular and soluble parameters in bronchoalveolar lavage fluid serves a potential use for diagnosing bronchiolitis obliterans.

Author	Method[a]	Rec.[b]	Stat.[c]	Cell differential characteristics					Lymphocyte subtypes					
				n*	Cell	MO	LY	NEU	n*	CD4+	CD8+	CD4/CD8	IL-2R+	HLADR+
Bronchiolitis Obliterans					*(10^3/ml)*	*(%)*	*(%)*	*(%)*						
Clelland[13]	2x50sal	-	median	14	150	70	8	27	-	14*10^3/ml	17*10^3/ml	-	20%	60%
			(range)	(24)	(10-840)	(13-100)	(0-24)	(0-87)	(5)	±21*10^3	±12*10^3	-	-	-
Ward[14]	3x60pbs	54	median	6	180	40	10	38	6	23%	57%	0.4	8%	45%
		(22-95)	(range)	(6)	(70-1910)	(6-55)	(2-41)	(16-87)	(6)	(12-75)	(20-71)	(0.31-3.7)	(4-14)	(18-63)
Ward[8]	3x60pbs	59	median	5	93	74	8	13	5	27%	55%	-	-	-
		(22-76)	(range)	(5)	(55-320)	(62-87)	(5-13)	(3-31)	(5)	(21-51)	(31-65)	-	-	-
Reynaud[15]	5x50pbs	-	mean	10	717	22.2	5.9	68.0	10	39.4%	29.2%	1.4	-	31%
			(±sd)	(27)	±175	±3.3	±1	±4	(12)	±3.6%	±2.5%	±0.1	-	±8.6%
Acute Rejection														
Clelland[13]	2x50sal	-	median	27	240	71	14	15	-	3*10^3/ml	33*10^3/ml	-	31%	60%
			(range)	(57)	(10-1650)	(23-98)	(0-60)	(0-72)	(11)	±3*10^3/ml	±43*10^3/ml	-	-	-
Whitehead[16]	3x60pbs	-	mean	-	-	61.8	25.7	7.8	-	-	-	1.1	-	15%
			±sd	(6)	-	±22.2	±22.5	±4.7	(6)	-	-	-	-	-
Crim[¶17]	3x50sal	-	mean	10	-	30	-	-	10	20%	61%	-	20%	-
			±sd	(32)	-	±4	-	-	(32)	±2%	±4%	-	±3%	-
Reynaud[15]	5x50pbs	-	mean	14	641	54.6	28.7	12.3	14	24%	48%	0.7	-	32.7%
			±sd	(58)	±81	±3.2	±2.5	±2.3	(51)	±1.9%	±2.7%	±0.1	-	±3.2%

TABLE I.

*Overview of available data in the literature on bronchoalveolar lavage fluid cell differential characteristics and lymphocyte subtypes in patients with bronchiolitis obliterans and acute rejection after lung transplantation. a: Method used for lavage (times x ml fluid instilled), sal=saline, pbs=phosphate buffered saline; b: Recovery of lavage fluid (%); c: statistics used; *n: number of patients who underwent lavage, between parenthesis the number of BAL procedures studied; - indicates data not presented in paper; *indicates data extracted from figures.*

INTRODUCTION

Bronchoalveolar lavage fluid (BALF) analysis serves an important clinical purpose in the detection of microbiological and viral complications after lung transplantation (LTX), yet detection of graft failure by using BALF cellular and soluble characteristics is still under investigation [1].

Chronic graft failure after LTX e.g. bronchiolitis obliterans (BO) is preferably detected as early in its development as possible (even before functional parameters like FEV_1 drop) in order to delay further progression by a change in the immunosuppressive regime [2,3]. This is important since an ongoing deterioration in health related quality of life occurs and only retransplantation is therapeutically available at the end [4,5]. However, early detection of BO is difficult. Whereas the diagnosis of acute rejection (AR) can be made reliably by using transbronchial biopsies, histopathological detection of early-stage BO lacks proper sensitivity [6,7]. BALF analysis may be helpful in this respect and might define which patients are at risk to develop BO in the long run [8-10].

At present, the exact pathophysiology of BO remains unknown. Airway neutrophilia seems to play an important role in the development of BO, and is associated with increased levels of interleukin-8 (IL-8), a neutrophil chemoattractant [11]. Since BO may result from an imbalance between tissue injury and repair leading to lung inflammation and fibrosis, other pro-inflammatory cytokines like interleukin-6 (IL-6) may be involved as well [12]. Furthermore, present studies report that lymphocytes may play an important role both in developing and established BO. BALF lymphocyte flow cytometric data in patients with BO and AR give no definite conclusions so far as to specific and sensitive indicators of BO after LTX (table 1). This may be due to low numbers of patients under study and the fact that the time after LTX has not been adequately adjusted for. Recently we have shown the importance of taking into account the time at which the bronchoalveolar lavage (BAL) is performed after LTX: total cell counts, numbers of $CD3^+$ cells, $CD4^+CD25^+$ cells and CD4/CD8 ratio all undergo an enormous change during the first two years after LTX [18].

In this study we searched for the feasibility of BALF analysis in detecting acute and chronic allograft rejection. We furthermore tried to obtain more insight into which lymphocyte population dominates AR and BO after LTX. BALF cellular differential characteristics, lymphocyte subtypes, IL-6 and IL-8 cytokine levels, were assessed in lung transplant patients and compared with patients without rejection or infection (good outcome), matched for the time after LTX. The study took place in a prospective LTX cohort in BALF samples from all patients transplanted in our centre between November 1990 and January 2000.

METHODS

Patients and selection of BALF samples

BALF-samples were assigned to AR, BO and two respectively matched good outcome (GO) LTX groups (GO-1 and GO-2). BALF samples were selected for inclusion in the AR group when acute rejection was confirmed by histopathological examination of transbronchial biopsies taken during the same procedure, BAL was performed before any AR-treatment was given, and when no other disease confounding factor (including the absence of BOS) was present [19]. BALF samples were selected for the BO group based on the bronchiolitis obliterans syndrome criteria as formulated by the ISHLT, and when no other disease confounding factor was present [20]. The GO-groups were selected when AR (histo-

	AR	GO-1	BO	GO-2
n*	37	59	48	50
Time after LTX, days*	92 (20-946)	95 (19-200)	372(92-1138)	359 (216-411)
Age#	44.1(12.3)	44.2(10.4)	43.9(12.3)	44.9(9.4)
Gender, M/F	17/20	31/28	27/21	26/24
FEV_1, %pred #	82.8(14.4)	100.6(15.6)	57.4(17.6)	98.1(17.5)
LTX type, n				
bilateral	26	45	39	42
unilateral	10	12	9	7
heart/lung	1	2	-	1
Pre LTX diagnosis, n				
COPD-AT	14	21	14	22
COPD	5	14	12	10
Bronchiectasis	6	6	5	2
Cystic Fibrosis	2	6	7	5
PPH	5	4	2	1
Secondary PH	2	3	3	4
PF	3	5	5	6

TABLE 2.
*Group characteristics of the included patients. AR: acute rejection patient group; BO: bronchiolitis obliterans patient group; GO-1: 'good outcome' patient group matched for time after LTX with the AR group; GO-2: 'good outcome' patient group matched for time after LTX with the BO group; COPD-AT: α_1-antitrypsin deficiency; PPH: primary pulmonary hypertension; PH: pulmonary hypertension; PF: pulmonary fibrosis; *median (range); #mean(SD).*

	AR	GO-1	*p*	BO	GO-2	*p*	*p(AR vs BO)*
Bronchial fraction (20ml PBS instilled)							
n*	27	48		40	44		
Recovery, %	31 (5-72)	26 (3-85)	*ns*	25 (5-64)	25 (8-73)	*ns*	*0.037*
Total cell, *10^3/ml	72 (10-1660)	157 (10-1280)	*ns*	140 (0-1750)	100 (0-510)	*ns*	*0.014*
AM, %	90 (7-96)	94 (23-98)	*0.049*	85 (0-96)	92 (61-100)	*0.001*	*ns*
LY, %	5 (0-32)	2 (0-42)	*0.026*	3 (0-20)	2 (0-54)	*ns*	*ns*
PMN, %	3 (0-89)	2 (0-73)	*ns*	9 (0-97)	2 (0-34)	*<0.0001*	*0.019*
EO, %	0 (0-1)	0 (0-2)	*ns*	0 (0-1)	0 (0-4)	*ns*	*ns*
No recovery, n	10	11		8	6		
Alveolar fraction (150 ml PBS instilled)							
n*	36	56		47	49		
Recovery, %	65.8 (9-94)	68 (28-83)	*ns*	59.3 (10-90)	70 (2-78)	*0.072*	*ns*
Total cell, *10^3/ml	141 (10-720)	225 (20-700)	*ns*	157 (11-1522)	125 (30-600)	*ns*	*ns*
AM, %	88 (61-98)	94 (76-99)	*<0.0001*	86 (1-96)	94 (50-99)	*<0.0001*	*ns*
LY, %	5 (0-37)	3 (0-22)	*<0.0001*	2.5 (0-35)	3 (0-44)	*ns*	*<0.0001*
PMN, %	1.5 (0-26)	2(0-11)	*ns*	9 (0-95)	2 (0-22)	*<0.0001*	*<0.0001*
EO, %	0.17 (0-6)	0.16(0-4)	*ns*	0.17 (0-3)	0.17 (0-5)	*ns*	*ns*
No recovery, n	1	3		1	1		

TABLE 3.
*Total and differential cell count in the bronchial- and alveolar fraction Results in patients with acute rejection (AR) or bronchiolitis obliterans (BO) compared with a 'good outcome' group matched for the time after LTX (respectively GO-1 & GO-2). Values given as median (range); AM: alveolar macrophages; LY: lymphocytes; PMN: neutrophils; EO: eosinophils; *n: number of lavages analysed; ns: not significant.*

logical or clinical suspicion) and BO (histopathological examination of transbronchial biopsies and/or decline in FEV_1 from baseline within two years after last BAL) were not present and when bacterial-, fungal-, CMV- or other viral infections were absent at the time of the BAL procedure. The time post-LTX at which the BAL procedure was performed, was divided in two periods (GO-1 and GO-2) for matching purposes with the AR and BO patient groups (table 2) [samples included from ref. 18]. All BALF samples used for analysis in this study for both the AR and BO patient group have not been used in previous studies. Single, bi-lateral and heart-LTX were performed according to established techniques [21].

Therapeutic protocol
Immunosuppression included up to 2-5 doses of antithymocyte globulins (rATG, Merieux, dosed at 3 mg/kg) the first 10 days post-LTX. The maintenance regimen consisted of cyclosporin (serum-through-levels: 400 μg/ml post-LTX, tapered in 3 weeks to 150 μg/ml), azathioprine (1-3 mg/kg/day) and prednisolone (0.1-0.2 mg/kg/day). Tacrolimus (serum level: 20 μg/L the first three weeks post-LTX, 15 μg/L between 3 weeks and 3 months and 10-12 μg/L thereafter) and mycophenolate mofetil (1000 mg twice daily) were not used in the first 8 years of our study. All patients received aciclovir and co-trimoxazole prophylaxis. There was no difference in the maintenance immunosuppressive regimen between the diagnostic groups.

Bronchoalveolar lavage and cell isolation
BAL was performed as described before [10,18]. This protocol was approved by the Medical Ethics Committee. The first 20ml portion was investigated for microbiology. The second 20ml portion was isolated as bronchial fraction (BF) and three 50ml fractions were pooled for the alveolar fraction (AF). BF and AF were immediately placed on ice (4°C) and further processed for leukocyte differentiation and flowcytometric analysis [18].

Diagnostic protocol
Diagnosis of bacterial-, fungal- or viral infections was made by obtaining positive cultures from the first BALF sample. CMV infection was assessed by CMV serology and CMV-antigenemia as described before [22,23].

Flowcytometric analysis (FACS)
Lymphocyte subtypes were measured using direct immunofluorescence with monoclonal antibodies as described before [18]. The following antibodies were used: $CD3^+$/$CD4^+$, $CD3^+$/$CD8^+$ (IQ-Products), $CD4^+$/$CD25^+$, HLADR/$CD4^+$, HLADR/$CD8^+$ (Beckton Dickinson, USA), $CD45RO^+$/$CD4^+$, $CD45RO^+$/$CD8^+$ (Dako, Denmark). Labelled cells were analysed using a FACS-Calibur (Beckton Dickinson, USA).

IL-6 & IL-8 measurements
IL-6 and IL-8 were measured by a chemiluminescent immunoassay (Immulite IL-6 & Immulite IL-8, Diagnostic Products Corp., Los Angeles, CA) performed on the Immulite system.

Statistical Analysis
Data were analysed using SPSS/PC^+ software (SPSS Benelux, Gorinchem, The Netherlands). Distributions of cell counts, lymphocyte subtypes and cytokine levels were compared between groups using the non-parametrical Mann-Whitney-U test. Receiver Operating Characteristic (ROC) curve analysis was used for calculation of predictive values. $P < 0.05$ was considered significant. Results are presented as median (range) unless otherwise mentioned.

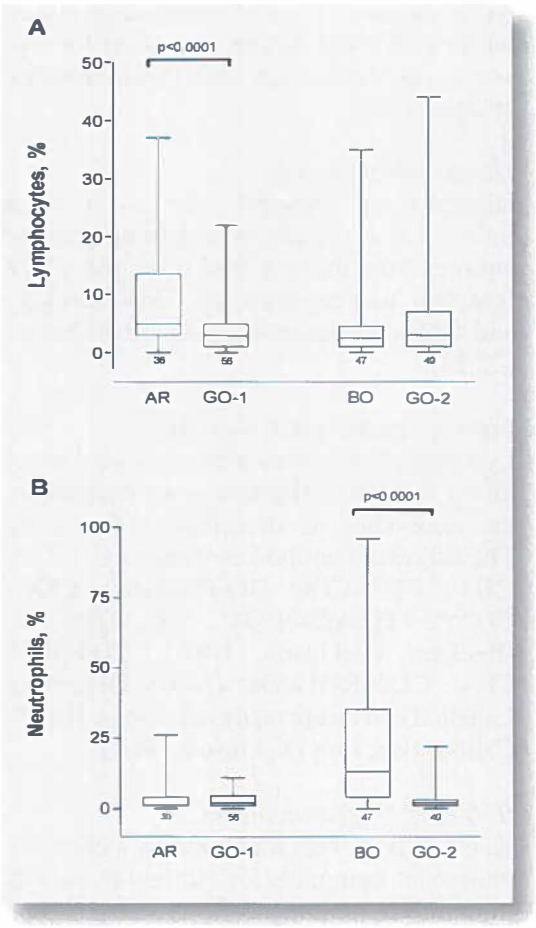

FIGURE 1A / 1B

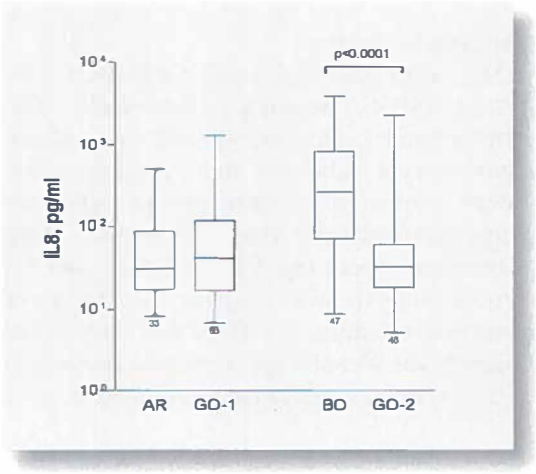

FIGURE 2

RESULTS

Patients

We included 37 patients with a diagnosis of AR at time BAL was performed and a 'time post LTX'-control group of 59 patients (GO-1). Forty-eight patients were included in the BO group at the time BAL was performed, and 50 patients were included in the 'time post LTX' matched control group (GO-2) (see table 2 for patient characteristics). The BO patient group consisted of 24 patients with BOS stage 1, 16 with BOS stage 2 and eight patients with BOS stage 3 [20].

Acute rejection

Recovery, total and differential cell count (table 3).

Similar to the BO patient group, there was no difference between the cellular characteristics of the BF and the AF in patients with acute rejection. There existed no difference in recovery between the AR and the 'good outcome' control group (GO-1). The BF results show a higher percentage of lymphocytes in patients with AR compared to their controls (GO-1): 5% (0-32) and 2% (0-42) respectively (p=0.026). The same result is

FIGURES 1A & B.
BALF lymphocyte and neutrophil percentage in AR and BO patients.
Boxplots indicate: range, 25th, 50th and 75th percentile of A: lymphocyte percentage and B: neutrophil percentage in BALF (alveolar fraction) of patients with acute rejection (AR) or bronchiolitis obliterans (BO) compared with a 'good outcome' group matched for the time after LTX (respectively GO-1 & GO-2). Numbers below the boxplots indicate the number of lavages analysed.

FIGURE 2.
BALF IL-8 concentration in AR and BO patients.
Boxplots indicate: range, 25th, 50th and 75th percentile of IL-8 concentration (logarithmic scale, pg/ml) in BALF (alveolar fraction) of patients with acute rejection (AR) or bronchiolitis obliterans (BO) compared with a 'good outcome' group matched for the time after LTX (respectively GO-1 & GO-2). Numbers below the boxplots indicate the number of lavages analysed.

shown in the AF: 5% (0-37) lymphocytes in the AR patient group and 3% (0-22) in the GO-1 group ($p<0.0001$) (figure 1). The total cell count did not reach a significant difference, but there was a trend towards a lower total cell count in both fractions for the AR group when compared to the control group (GO-1).

Lymphocyte subtypes (alveolar fraction only, table 4) and cytokines

There were no differences in any of the lymphocyte subtypes, IL-8 and IL-6 cytokine levels between patients with AR and controls (GO-1) (IL-8: 30.9 pg/ml (8-504) for AR and 41.9 pg/ml (7-1300) for GO-1 (ns) (figure 2); IL-6: 8.2 pg/ml (0-17.2) for AR and 8.6 (0-84.3) for GO-1 (ns)).

Predictive values

The predictive values of the BAL lymphocyte percentage for the diagnosis of AR are shown in table 5.

Bronchiolitis obliterans

Recovery, total and differential cell count (table 3)

There was no difference between the cellular characteristics of the BF and the AF in BO patients. No difference was found in recovery between BO and the 'good outcome' control group (GO-2). BO patients had

	AR	GO-1	*p*	BO	GO-2	*p*	*p(AR vs BO)*
FACS, n*	29	37		27	32		
$CD3^+$, %	84 (42-96)	87 (67-98)	*ns*	88 (25-97)	86 (49-98)	*ns*	*ns*
$CD4^+$,%	23 (10-54)	21 (7-70)	*ns*	24 (5-49)	25 (10-67)	*ns*	*ns*
$CD8^+$,%	52 (16-79)	59 (23-74)	*ns*	54 (11-95)	48 (17-78)	*ns*	*ns*
CD4/CD8 ratio	0.44 (0.17-2.70)	0.37 (0.09-1.54)	*ns*	0.54 (0.1-1.34)	0.57 (0.15-2.15)	*ns*	*ns*
$CD4^+$ $HLADR^+$, %	59 (13-85)	55 (11-93)	*ns*	58 (42-99)	50 (14-99)	*0.062*	*ns*
$CD8^+HLADR^+$,%	60.5 (10-85)	54 (13-99)	*ns*	62 (30-100)	45 (13-99)	*0.054*	*ns*
$CD4^+CD45^+$,%	100 (94-100)	100 (94-100)	*ns*	100 (91-100)	100 (89-100)	*ns*	*ns*
$CD8^+CD45^+$,%	92 (78-100)	94 (76-100)	*ns*	95.5 (74-100)	87 (53-100)	*0.012*	*ns*
$CD4^+CD25^+$,%	29 (4-91)	33 (0-86)	*ns*	30.5 (2-66)	18 (1-54)	*0.024*	*ns*
$CD19^+$,%	1 (0-8)	1 (0-4)	*ns*	1 (0-8)	1 (0-12)	*ns*	*ns*
NK,%	6 (0-51)	4 (0-24)	*ns*	4 (0-13)	5 (0-41)	*ns*	*ns*

TABLE 4.

*Lymphocyte subtypes in the alveolar fraction. Results in patients with acute rejection (AR) or bronchiolitis obliterans (BO) compared with a 'good outcome' group matched for the time after LTX (respectively GO-1 & GO-2). Values given as median (range). *n: number of lavages analysed; ns: not significant; NK: Natural Killer cells.*

CUT-OFF POINT		Sensitivity, %	Specificity, %	PPV, %	NPV, %
Acute rejection					
Lymphocytes, %	≤ 1	40.4 (26.4-55.7)	95.6 (84.8-99.3)	90.5*	60.6*
Bronchilitis obliterans					
Neutrophils, %	> 3	87.0 (73.7-95.0)	77.6 (63.4-88.2)	78.4#	86.4#
IL-8, pg/mL	> 71.4	74.5 (59.6-86.0)	83.3 (69.8-92.5)	81.4#	76.9#

TABLE 5.

*Predictive values of BAL lymphocytes for acute rejection and BAL neutrophils and IL-8 levels for bronchiolitis obliterans. Sensitivity and specificity given as percentage (95% confidence interval). PPV: positive predictive value; NPV: negative predictive value. * based upon our own study population; # based on a BO prevalence of 50% [37].*

a higher percentage of neutrophils in BF and AF than the GO-2 group patients: 9% (0-97) versus 2% (0-34) for BF ($p<0.0001$), and 9% (0-95) versus 2% (0-22) in AF respectively ($p<0.0001$) (figure 1). Due to the increase in percentage of neutrophils in patients with BO a significant lower percentage of AM was found in both fractions as well. Total cell count was not different between the BO and GO-2 patient groups in both the BF and AF.

Lymphocyte subtypes
(alveolar fraction only, table 4)
$CD4^+CD25^+$ lymphocyte percentage was higher in BO when compared to their controls ($p<0.05$). Furthermore a higher percentage $CD8^+CD45^+$ was shown between BO and controls ($p<0.05$). Other lymphocyte subtypes did not show any significant difference, although a tendency was shown for higher levels of HLA-DR expression of both $CD4^+$ and $CD8^+$ cells in patients with BO.

Cytokines
(alveolar fraction only)
IL-8 in BALF was significantly higher in BO patients (265 pg/ml (8-3802)) than in their controls (GO-2: 32.5 pg/ml (5-2247), $p<0.0001$) (figure 2). No difference in IL-6 levels was found between the BO and GO-2 group, 7.7 pg/ml (0-105) and 7.2 pg/ml (0-28.8) respectively. Furthermore IL-8 correlated significantly with the neutrophil percentage in both the BF ($r=0.588$, $p<0.0001$) and the AF ($r=0.602$, $P<0.0001$).

Predictive values
When using the ROC curve analysis, the most optimal cut-off point for the BAL neutrophil percentage was >3% with respect to the presence of BO. The cut-off point for IL-8 was >71.4 pg/mL. The predictive values are shown in table 5.

DISCUSSION

This study aimed to assess the feasibility of BALF analysis in the detection of bronchiolitis obliterans and acute rejection taking into account a control group of LTX patients who had a good outcome, matched for the time after LTX. We showed a clear difference between BO patients and their controls, in that higher numbers of neutrophils and a high BALF IL-8 concentration are predictive for the diagnosis of BO. Furthermore, there are higher $CD4^+CD25^+$ and $CD8^+CD45^+$ lymphocyte percentages in patients with BO. The BALF analysis can discriminate AR patients from stable LTX patients by a higher lymphocyte count. However, analysis of lymphocyte subtypes in AR did not show difference, suggesting that all lymphocyte subtypes are similarly increased in number.

The strength of the study is that our results are not confounded by concomitant frequently occurring co-morbidities of lung transplant recipients. Observations were done in absence of any other complication of the airways such as bacterial, viral or fungal infectious disease, and in the case of AR no concurrent BO and vice versa. A major methodological advantage of our study is that we selected two separate control groups for BO and AR respectively, since they occur at different time points after LTX. As shown before it is very important to take into account the time at which the BAL is performed after LTX when comparing BALF cellular characteristics [18]. The total cell count, $CD3^+$, $CD4^+$, $CD4^+CD25^+$ and especially $CD8^+$ lymphocyte counts all undergo an extensive change in stable LTX patients during the first two years after LTX [18].

In the current study, both the alveolar and the bronchial fraction showed comparable results of BALF analyses. This contrasts to the observations in patients who have

normal FEV_1 at time of BAL, but who are at risk for development of BO later on. In this case cellular contents of the bronchial fraction, which is assumed to represent the proximal airways [24], is superior for prognosis prediction to the more peripheral collected alveolar fraction [10].

BALF in AR

Acute rejection is in our study characterised by a marked BALF lymphocytosis, in the presence of a normal total cell count. As has been shown before, the severity of the histopathological rejection grade is not associated with the degree of the lymphocytosis found in BALF [13]. Also has BALF lymphocytosis a low sensitivity for AR, but a fairly good specificity, which makes AR unlikely when the lymphocyte percentage is ≤ 1% (table 5). That BALF lymphocytosis is present during acute rejection is not surprising because soon after implantation the lung is infiltrated by both $CD4^+$ and $CD8^+$ T-cells and during acute rejection episodes the infiltration into the airways of these cells becomes more prominent [25].

All lymphocyte subtypes studied in our analysis showed no differences at all between patients with AR and the good-outcome patients. This finding is supported by Crim and co-workers who in a more limited evaluation also found no difference between AR and stable LTX patients with respect to $CD4^+$ and $CD4^+IL\text{-}2R^+$ cells in BALF [17]. The slightly more pronounced $CD8^+$ percentage found in their study could not be confirmed by our results. Clelland and co-workers found in their evaluation of lymphocyte phenotypes in AR a higher $CD4^+$ and $CD8^+$ cell count in transbronchial biopsies compared to findings in stable LTX patients [13]. When they would have had the ability to correct their findings for time after LTX at which the BAL was performed, there might have been no differences at all, presuming the bronchoscopies in the AR patients were performed early after LTX compared to the later performed bronchoscopies in stable patients [18].

IL-6 and IL-8 concentrations were similar in AR patients and stable LTX patients in our study. It has been shown that pro-inflammatory cytokine levels increase during allograft rejection, e.g. IL-1 and TNF-α and most importantly IL-2. Furthermore it has been suggested that alveolar macrophages (AM) produce more IL-6 in patients with AR than in stable patients after LTX [26,27]. Due to the dilutive effect of the BAL procedure, it is probable that BALF analysis is not sensitive enough to detect this higher IL-6 production, in contrast to the higher IL-6 production as measured after incubation of cultured cells for 24hrs [12,26].

BALF in BO

Our results indicate, when compared to a properly selected control group, that BO is characterised at time of diagnosis by a marked BALF neutrophilia in the presence of a normal total cell count and a marked elevation in IL-8 concentration. This finding confirms previous reports showing the participation of neutrophilic granulocytes in the process of airway fibrosis and necrosis leading to BO [9,28-30]. In this study we also showed fairly acceptable predictive values for both the neutrophil percentage as well as the height of IL-8 in the BALF with respect to BO (table 5). However, one has to be aware that these interpretations have been made in BALF samples without concurrent infectious disease or acute rejection.

In a previous study [10], slightly favourable odds ratios for the BF compared to the AF for predicting future BO might be present. In the current study we were not able to detect a difference between the more centrally sampled BF (first 20 mL) and the assumed

alveolar sampled AF (second 150 mL). This may be due to the already overwhelming neutrophilia present in the airways as well as the fact that the AF is being aspirated after having passed the small airways (thus the BF site).

Ward et al. have previously shown that higher numbers of $CD4^+$ and $CD8^+$ lymphocytes exist in BALF of stable LTX patients, when compared to data of normal healthy volunteers. Until now no agreement exists in the literature on BALF lymphocyte flow-cytometric data in BO (table 1). Our results, with a proper choice of control group, show that there are no differences between BO and their stable LTX controls for the main lymphocyte subtypes $CD3^+$, $CD4^+$ and $CD8^+$. These results are supported by previous findings in transbronchial biopsies and in a small population investigated with BALF (n=6) [13,14].

Whereas in stable patients, the $CD4^+CD25^+$ percentage decreases sharply after the first months after LTX [18], the current study shows a higher percentage of $CD4^+CD25^+$ lymphocytes in patients with BO compared to controls, possibly indicating an ongoing allograft response. The single previous study in LTX patients showed no difference when assessing $CD25^+$ cells in BALF, investigated in a small group of BO patients (n=6) and control LTX patients who were not matched for time after LTX [8]. Furthermore we found a slightly elevated $CD8^+CD45^+$ lymphocyte percentage in BO, with a trend observed in a higher $HLA\text{-}DR^+$ of both the $CD4^+$ and $CD8^+$ cells. In stable patients after LTX we have previously shown that the lymphocyte activation and memory markers show a minor decrease during the first years post LTX [18]. Thus in general, the BALF lymphocytes in BO patients seem more activated when compared to patients with good outcome after LTX [8,18,31].

The high IL-8 concentration in BALF we observed in BO patients correlates with the neutrophil count. In the airways, IL-8 is mainly produced by alveolar macrophages and respiratory epithelial cells [32]. On-going epithelial damage may constitute a possible source of the very high IL-8 levels measured. This epithelial damage can occur as a result of a number of pathogenic factors known to be involved in BO development, such as allograft reactivity (including HLA-mismatch), chronic infectious pressure (including CMV), acute rejection episodes, graft ischaemic time, and possibly also the maintenance immunosuppressive regime used [33,34]. At an early stage of BO, e.g. when small airway changes are present yet without a decline in FEV_1, slightly elevated IL-8 levels and neutrophil counts can be found [11]. As shown by our data, once BO is present and ongoing tissue scarring occurs, airway neutrophilia, possibly mediated by IL-8, dominates BO [9,11,30].

Data so far published present a dynamic profile of cytokines in BALF of BO patients from just post-LTX until the moment this disease becomes clinically manifest. Our previous study showed that high BALF levels of IL-6 and MCP-1 can be found in the first months post-LTX, accompanied by normal IL-8 levels in patients proven to later develop BO [10]. The current study extends these observations in that IL-6 shows normal levels once BO is present. Thus IL-6 possibly plays an important role in the early stages of BO development as a pro-inflammatory cytokine, presumably released by respiratory epithelial cells and alveolar macrophages [12,33]. IL-6 has been shown to prolong the lifetime and activation of T-lymphocytes thereby increasing the alloreactivity between T-lymphocytes and epithelial cells. Recently we showed that the interaction of these alloreactive host-lymphocytes with airway epithelial cells of the donor resulted in abundant

cytokine production of the epithelial cells [35]. Once BO is present IL-8 production is still enhanced and this epithelial derived pro-inflammatory cytokine acts as a neutrophilic chemo-attractant as shown by the high number of neutrophils present in BO patients [33,36].

Our study shows a possible diagnostic role for cellular and soluble analysis of BALF, since it may detect BO and AR after LTX. BALF neutrophilia and an elevated IL-8 is very suggestive of established BO. When BALF analysis shows a lymphocytosis, one should beware of AR. BALF lymphocyte subtypes are already highly activated in stable patients after LTX, and this becomes even more pronounced in BO, but not in the case of AR. BALF analysis can thus give us better insight into the pathophysiological mechanisms underlying acute and chronic allograft rejection. A search for more specific BALF markers related to these pathological conditions remains needed, in order to detect graft failure as early and sensitively as possible.

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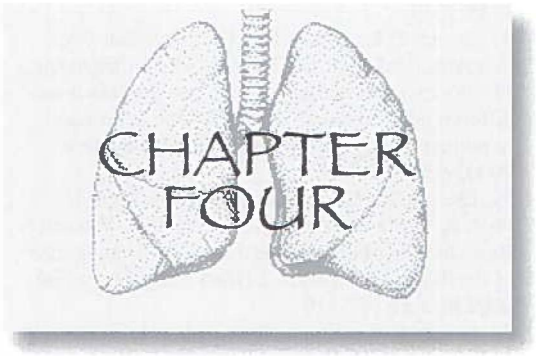
REFERENCES

1. Tiroke AH, Bewig B, Haverich A. Bronchoalveolar lavage in lung transplantation. State of the art. Clin Transplant 1999;13:131-157
2. Adams BF, Berry GJ, Huang X, Shorthouse R, Brazelton T, Morris RE. Immunosuppressive therapies for the prevention and treatment of obliterative airway disease in heterotopic rat trachea allografts. Transplantation 2000;69:2260-6
3. Revell MP, Lewis ME, Llewellyn-Jones CG, Wilson IC, Bonser RS. Conservation of small-airway function by tacrolimus/cyclosporine conversion in the management of bronchiolitis obliterans following lung transplantation. J Heart Lung Transplant 2000;19:1219-23
4. Berg vd JWK, Geerstma A, Bij vd W, Koëter, GH, Boer de WJ, Postma, DS, and Ten Vergert EM. Bronchiolitis obliterans syndrome after lung transplantation and health-related quality of life. Am J Respir Crit Care Med 2000;161:1937-1941
5. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, and Novick RJ. The registry of the international society for heart and lung transplantation: seventeenth official report. J Heart Lung Transplant 2000;19:909-931
6. Chan CC, Abi-Saleh WJ, Arroliga AC, Stillwell PC, Kirby ThJ, Gordon SM, Petras RE, Metha AC. Diagnostic yield and therapeutic impact of flexible bronchoscopy in lung transplant recipients. J Heart Lung Transplant 1996;15:196-205
7. Guilinger RA, Paradis IL, Dauber JH, Yousem SA, Williams PA, Keenan RJ, Griffith BP. The importance of bronchoscopy with transbronchial biopsy and bronchoalveolar lavage in the management of lung transplant recipients. Am J Respir Crit Care Med 1995;152:2037-43
8. Ward C, Whitford H, Snell G, Bao H, Zheng L, Reid D, Williams TJ, Walters EH. Bronchoalveolar lavage macrophage and lymphocyte phenotypes in lung transplant recipients. J Heart Lung Transplant, 2001;20:1064-1074
9. Zheng L, Walters EH, Ward C, Wang N, Orsida B, Whitford H, Williams TJ, Kotsimbos T, Snell GI. Airway neutrophilia in stable and bronchiolitis obliterans syndrome patients following lung transplantation. Thorax 2000;55:53-59
10. Scholma J, Slebos DJ, Boezen HM, Berg vd JWK, Bij vd W, Boer de W, Koëter GH, Timens W, Kauffman HF, and Postma DS. Eosinophilic granulocytes and interleukin-6 level in bronchoalveolar lavage fluid are associated with the development of obliterative bronchiolitis after lung transplantation. Am J Respir Crit Care Med 2000;162:2221-2225
11. DiGiovine B, Lynch III JP, Martinez FJ, Flint A, Whyte RI, Iannettoni MD, Arenberg DA, Burdick MD, Glass MC, Wilke CA, Morris SB, Kunkel SL, Strieter RM. Bronchoalveolar lavage neutrophilia is

associated with obliterative bronchiolitis after lung transplantation: role of IL-8. J Immunol 1996;157: 4194-42
12. Magnan A, MegeJL, Escallier JC, Brisse J, Capo C, Reynaud M, Thomas P, Meric B, Garbe L, Badier M, Viard L, Bongrand P, Giudicelli R, Metras D, Fuentes P, Vervloet D, Noirclerc M. Balance between alveolar macrophage IL-6 and TGF-beta in lung transplant recipients. Am J Respir Crit Care Med 1996;153:1431-1436
13. Clelland C, Higenbottam T, Stewart S, Otulana B, Wreghitt T, Gray J, Scott J, and Wallwork J. Bronchoalveolar lavage and transbronchial biopsy during acute rejection and infection in heart-lung transplant patients. Am Rev Respir Dis 1993;147: 1386-1392
14. Ward C, Snell GI, Zheng L, Orsida B, Whitford H, Williams TJ, Walters EH. Endobronchial biopsy and bronchoalveolar lavage in stable lung transplant recipients and chronic rejection. Am J Respir Crit Care Med 1998;158:84-91
15. Reynaud-Gaubert M, Thomas P, Gregoire R, Badier M, Cau P, Sampol J, Giudicelli R, Fuentes P. Clinical utility of bronchoalveolar lavage cell phenotype analyses in the postoperative monitoring of lung transplant recipients. Eur J Cardiothorac Surg 2002 Jan;21:60-6
16. Whitehead BF, Stoehr C, Finkle C, Patterson G, Theodore J, Clayberger C, and Starnes VA. Analysis of bronchoalveolar lavage from human lung transplant recipients by flow cytometry. Respir Med 1995;89:27-34
17. Crim C, Keller C, Dunphy C, Maluf H, Ohar J. Flow cytometric analysis of lung lymphocytes in lung transplant recipients. Am J Respir Crit Care Med 1995;153:1041-1046
18. Slebos DJ, Scholma J, Boezen HM, Koëter GH, van der Bij W, Postma DS, Kauffman HF. Longitudinal profile of bronchoalveolar lavage cell characteristics in patients with a good outcome after lung transplantation. Am J Respir Crit Care Med 2002;165:501-507
19. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Tazelaar HD. Revision of the 1990 Working Formulation for the Classification of Pulmonary Allograft Rejection: Lung Rejection Study Group. J Heart Lung Transplant 1996;15:1-15
20. Cooper, J. D., M. E. Billingham, T. Egan, M. I. Hertz, T. L. Higenbottam, J. Lynch, J. Mauer, I. Paradis, G. A. Patterson, C. Smith, E. P. Trulock, C. Vreim, and S. Yousem. A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation. J Heart Lung Transplant. 1993;12:713-716
21. Cooper JD. The evolution of techniques and indications for lung transplantation. Ann Surg 1990;212: 249-255
22. Van der Bij W, Torensma R, Van Son WJ, Anema J, Schirm J, Tegzess AM, The TH. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leucocytes. J Med Virol 1988;25:179-188
23. Van der Giessen M, van den Berg AP, W. van der Bij, Postma DS, van Son WJ, The TH. Quantitative measurement of CMV-specific IgG- and IgM-antibodies in relation to CMV antigenemia and disease activity in kidney recipients with an active CMV infection. Clin Exp Immunol 1990;80:56-61
24. Kelly CA, Kotre CJ, Ward C, Hendrick DJ, Walthers EH. Anatomical distribution of bronchoalveolar lavage fluid as assessed by digital subtraction radiography. Thorax 1987;42:624-628.
25. King-Biggs MB. Acute pulmonary allograft rejection. Clinics in chest medicine. 1997;18(2): 301-310
26. Magnan A, Mege JL, Reynaud M, Thomas P, Capo C, Garbe L, Meric B, Badier M, Bongrand P, and Viard L. Monitoring of alveolar macrophage production of tumor necrosis factor- alpha and interleukin-6 in lung transplant recipients Am J Respir Crit Care Med 1994;150:684-689
27. Rizzo M, SivaSai KSR, Smith MA, Trulock EP, Lynch JP, Patterson A, Mohanakumar T. Increased expression of inflammatory cytokines and adhesion molecules by alveolar macrophages of human lung allograft recipients with acute rejection: decline with resolution of rejection. J Heart Lung Tranplant 2000;19:858-865
28. Snell G, Ward C, Wilson J, Orsida B, Williams T, Walters E. Immunopathological changes in the airways of stable lung transplant recipients. Thorax 1997;52:322-328
29. Riise GC, Kjellstrom C, Ryd W, Schersten H, Nilsson FN, Martensson G, Andersson BA. Inflammatory cells and activation markers in BAL during acute rejection and infection in lung transplant recipients: a prospective, longitudinal study. Eur Respir J 1997;10:1742-1746
30. Riise GC, Williams A, Kjellstrom C, Schersten H, Andersson BA, Kelly FJ. Bronchiolitis obliterans syndrome in lung transplant recipients is associated with increased neutrophil activity and decreased anti-oxidant status in the lung. Eur Respir J 1998;12: 82-88
31. Yousem SA, Curley JM, Dauber J, Paradis I, Rabinowich H, Zeevi A, Duquesnoy R, Dowling R, Zenati M, Hardesty R, Griffith BP. HLA-class II antigen expression in human heart-lung allografts. Transplantation 1990;49:991-995
32. Baggiolini M, Walz A, Kunkel SL. Neutrophil activating peptide-1/Interleukin-8, a novel cytokine that activates neutrophils. J Clin Invest 1989;84:

1045-1049
33. Borger P, Kauffman HF, Timmmerman JA, Scholma J, vd Berg JW, Koëter GH. Cyclosporine, FK506, mycophenolate mofetil, and prednisolone differentially modulate cytokine gene expression in human airway derived cells. Transplantation 2000;69: 1408-1413
34. Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M, Mallory GB, Snell GI, Yousem S. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002 Mar;21:297-310
35. Borger P, Kauffman HF, Scholma J, Timmerman JAB, Koëter GH. Human allogenic $CD2^+$ lymphocytes activate airway derived epithelial cells to produce Il-6 and IL-8. Possible role for the epithelium in chronic allograft rejection. J Heart Lung Transplant 2002;21:567-575
36. Elssner A, Jaumann F, Dobmann S, Behr J, Schwaiblmair M, Reichenspurner H, Furst H, Briegel J, and Vogelmeier C. Elevated levels of interleukin-8 and transforming growth factor-beta in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells. Transplantation 2000;70:362-367
37. Estenne M,.Hertz MI. Bronchiolitis obliterans after human lung transplantation. Am J Respir Crit Care Med 2002;166:440-444



Eosinophilic granulocytes and IL-6 level in bronchoalveolar lavage fluid are associated with the development of obliterative bronchiolitis after lung transplantation

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Abstract

In a prospective cohort study we assessed whether changes in total cell counts and differentiation and IL-6, IL-8 and MCP-1 levels in bronchoalveolar lavage fluid (BALF) are associated with a higher risk to develop obliterative bronchiolitis (OB). We investigated 60 lung transplant patients (follow up of 2 to 8 years) with either histological evidence of OB within one year after lung transplantation (n=19) or no pathology, good outcome (GO) for at least 24 months and well preserved lung function, i.e. FEV_1 ≥ 80% of baseline (n=41). Median time between lung transplantation and the first BAL was 42 days for the GO group and 41 days for the OB group ($p>0.05$). In the bronchial fraction, median total cell counts (0.06 x10^3/ml vs 0.04 x10^3/ml), lymphocyte (9 x10^3/ml vs 2 x10^3/ml), and eosinophilic granulocyte counts (1 x10^3/ml vs 0) were significantly higher in the OB group than in the GO group ($p<0.05$). In the alveolar fraction, this was the case for the median value of neutrophilic granulocyte counts (19 x10^3/ml vs 4 x10^3/ml) respectively. Median values of IL-6 and IL-8 levels in both bronchial (IL-6: 23 vs 6 pg/ml, IL-8: 744 vs 102 pg/ml) and alveolar fractions (IL-6: 13 vs 3 pg/ml, IL8: 110 vs 30 pg/ml) of the BALF were significantly higher in the OB group than in the GO group. By means of logistic regression we showed that higher total cell, neutrophilic granulocyte and lymphocyte counts, the presence of eosinophilic granulocytes, and higher levels of IL-6 and IL-8 were significantly associated with an increased risk to develop OB. We conclude that monitoring cell counts, neutrophilic- and eosinophilic granulocytes, IL-6 and IL-8 in BALF within 2 months after lung transplantation in addition to the TBB pathology will contribute to a better identification and management of the group of patients at risk for developing OB within a year.

INTRODUCTION

Lung transplantation is generally accepted as therapy for several end-stage pulmonary diseases. Long-term survival of lung transplant patients is limited by the development of chronic transplant dysfunction. This condition affects at least 30 to 40% of the patients within the first years after lung transplantation, is nearly always irreversible, and is the most common cause of death 1 yr after lung transplantation (1). Clinically, chronic transplant dysfunction is associated with bronchiolitis obliterans syndrome (BOS). BOS is defined as a 20% or larger decrease in the forced expiratory volume in one second (FEV_1) from the baseline FEV_1 of the recipient, whereas other possible causes of deterioration of graft function, such as infection, have to be excluded (2). Histologically, chronic transplant dysfunction is represented by obliterative bronchiolitis (OB) and vasculopathy in lung tissue. OB is characterized by submucosal scarring that causes partial or total obliteration of membranous and respiratory bronchioles (3).

Usually, when chronic transplant dysfunction is suspected, a bronchoscopy is performed to exclude other causes of the deterioration of graft function, such as infection, problems with anastomoses, or acute rejection. The sensitivity of transbronchial lung biopsy (TBB) in detecting OB varies but is generally low (4). A more definitive diagnosis can be made by the use of open lung biopsy, but the drawback of this sensitive method is the supposed increased risk of associated morbidity. Bronchoalveolar lavage (BAL), a less invasive technique than TBB, is commonly accepted for detecting infections. Several studies have investigated the usefulness of BAL in providing specific information related to chronic transplant dysfunction but did not focus on finding early predictors for the development of OB. At present, the exact pathophysiology mechanisms of chronic transplant dysfunction and the development of OB are unknown, and diagnostic markers for predicting the development of OB at an early timepoint are poor. Accumulating evidence suggests that airway neutrophilia observed in the lungs plays an important role in the development of OB (7). Moreover, the neutrophilic granulocyte is the only cell that correlates with OB development and is associated with interleukin-8 (IL-8) concentrations, where IL-8 seems to act as neutrophil chemoattractant (10, 11). Because OB may be the result of dysbalance between tissue injury and repair leading to inflammation and fibrosis of the lungs, we postulate that also other proinflammatory and profibrotic cytokines such as IL-6 and monocyte chemoattractant protein-1 (MCP-1) may be involved. IL-6 has been shown to possess profibrotic properties in relation with tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) (12), and IL-6 production by epithelial cells and alveolar macrophages is one of the mechanisms by which local pulmonary inflammatory processes like acute rejection and infectious lung diseases are stimulated (13, 14). The CC-chemokine MCP-1 is a potent monocyte chemoattractant, which is expressed by airway epithelial cells. Accelerated MCP-1 release is seen in chronic inflammatory pulmonary diseases such as idiopathic pulmonary fibrosis (IPF) and sarcoidosis. Monocytes may stimulate fibrinogenic mediator release which enhances tissue remodeling. Recently, MCP-1 has been described as a potential inflammatory mediator in OB (15).

It may be proposed that some of the patients are more prone to an enhanced inflammatory response after lung transplantation, ultimately resulting in OB. Therefore, in this prospective cohort study, we investigated the early relation between changes in leukocyte and differential counts, IL-6, IL-8, and MCP-1 concentations in BAL

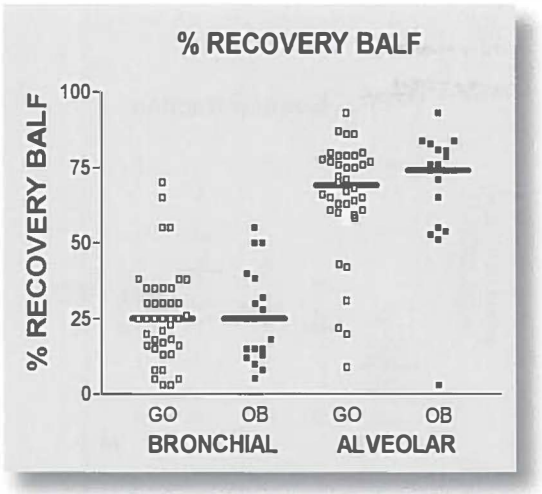

FIGURE 1.

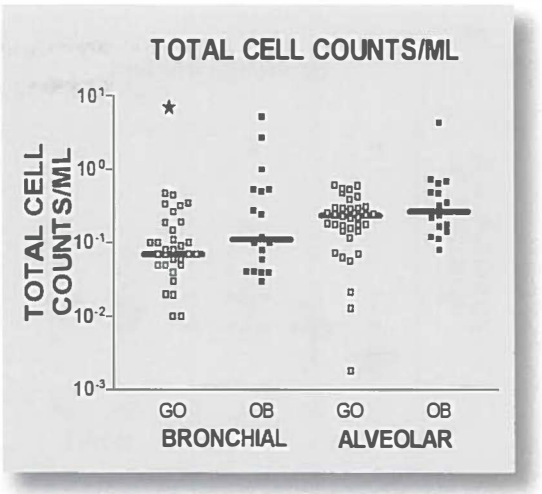

FIGURE 2.

fluid (BALF) and the development of OB in 60 lung transplant patients with a follow-up of 2 to 8 yr.

METHODS

Patients and Study Design

A prospective cohort study including 60 patients who underwent lung transplantation between November 1990 and September 1996 was carried out using BALF samples. Informed consent was obtained from the patients. The study group consisted of patients who had histologic evidence of OB within 1 yr after lung transplantation (n = 19). The diagnosis of OB was based on a classification and grading of pulmonary transplant dysfunction promulgated by the International Society for Heart and Lung Transplantation (3).

Control patients with good outcome (GO) were patients who did not develop OB or other pathology for at least 24 mo (43 mo median; range: 24 to 96 mo) after lung transplantation and had well-preserved lung function, i.e., $FEV_1 \geq 80\%$ of baseline value (n = 41). Single and bilateral lung transplantations were performed according to established techniques and criteria described earlier (16).

FIGURE 1.
Recovery of Bronchoalveolar lavage fluid of lung transplant patients who developed obliterative bronchiolitis (OB) or not (termed good outcome, GO)
GO = Good outcome group; OB = Obliterative Bronchiolitis group; Dash = median value.

FIGURE 2.
Total cell counts in BALF of lung transplant patients who developed obliterative bronchiolitis (OB) or not (termed good outcome, GO)
GO = Good outcome group; OB = Obliterative Bronchiolitis group; Dash = median value.
** significant difference ($p<0.05$) between GO and OB group*

Diagnostic Protocol and Follow-up

Graft function was determined by formal spirometry (at least twice weekly during hospitalization and at every outpatient visit) and by extensive pulmonary function assessment (volume-flow measurements, diffusion capacity, pulmonary exercise testing), i.e., before discharge and every 6 mo after lung transplantation. In addition, daily spirometry with a pocket spirometer (17) was carried out at home. Acute allograft rejection was diagnosed clinically in case of deteriorating pulmonary function without

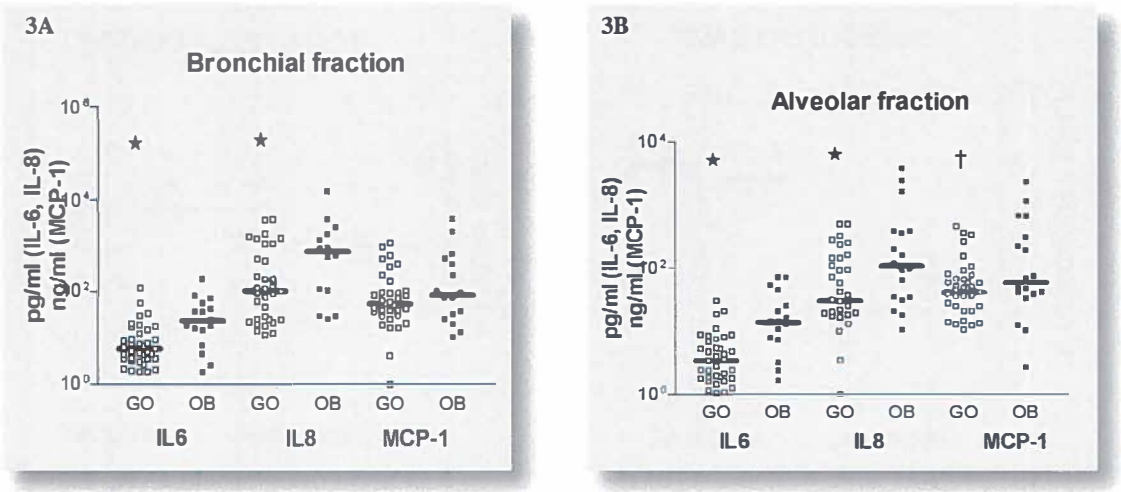

FIGURE 3A, 3B.
*Cytokine levels in BALF of lung transplant patients who developed obliterative bronchiolitis (OB) or not (termed good outcome, GO). GO = Good outcome group; OB = Obliterative Bronchiolitis group; Dash = median value. * significant difference ($p<0.05$) between GO and OB group; † tendency toward difference ($p<0.1$) between GO and OB group*

infection and with a positive response on high-dose methylprednisolone. The histologic diagnosis was defined according to Yousem and coworkers (3). Cytomegalovirus (CMV) infection was monitored by CMV serology (18) and testing for CMV antigenemia (19) as described before. Active infection was defined as the presence of CMV antigenemia or when a significant rise of CMV-specific antibodies occurred.

Therapeutic Protocol

Immunosuppression induction included up to five gifts of antithymocyte globulins (ATG) in the first 10 d after transplantation. The maintenance immunosuppressive regimen consisted of cyclosporine, azathioprine (1 to 3 mg/kg/d), and prednisolone (0.1 to 0.2 mg/kg/d). Cyclosporine administration was aimed at a trough level of 400 ng/ml at the start, as determined by high-performance liquid chromatography, which was tapered in 3 wk to 150 ng/ml. Acute rejection was treated with a 3-d course of 500 to 1,000 mg methylprednisolone intravenously daily. In case of persistent transplant dysfunction or when OB was diagnosed, cytolytic therapy with rATG was started. During this study, the newer immunosuppressive drugs, tacrolimus and mycophenolate mofetil, were not yet available. All patients received aciclovir 4 daily doses of 200 mg orally for herpes prophylaxis, and co-trimoxazole 960 mg orally on alternative days for *Pneumocystis carinii* prophylaxis.

BAL and Cell Isolation

BAL and bronchoscopy were carried out according to our protocol (20) at the same intervals as the pulmonary function measurements. This protocol was approved by the Medical Ethical Committee. BAL and bronchoscopy were carried out, 1 mo and every 6 mo after lung transplantation, and in the event of clinical indication. A fiberoptic bronchoscope was placed in wedge position in one of the segments of usually the right middle lobe, and 2 aliquots of 20 ml and 3 aliquots of 50 ml prewarmed phosphate-buffered saline (PBS) were instilled.

The first 20-ml portion was investigated for viruses, bacteria, and fungi. The second 20-ml portion (bronchial fraction) was isolated and studied separately, whereas the other 50-ml fractions were pooled (alveolar fraction) (21, 22). Bronchial and alveolar fraction were immediately placed on ice (4° C), filtered through a nylon gauze, and centrifuged (Heraeus-Sepatech, Osterode, Germany) for 5 min at 400 g and 4° C to remove cells and debris. BALF was decanted and stored in small portions at 80° C. BAL cells were washed two times with PBS/ 0.1% (wt/vol) glucose (Merck, Amsterdam, The Netherlands). Viability was tested by incubation of 10^6 cells/ml with trypan blue solution (1:1) (vol:vol) (Gibco, Life Technology, Breda, The Netherlands) for 5 min. Viability was measured by determining the percentage of unstained cells in duplicate.

Biopsies
TBB (usually 5 to 10 at each procedure) were taken under fluoroscopic control using forceps No. 21 and/or 15c ("alligator"; Olympus, Tokyo, Japan). TBB were evaluated histopathologically.

Leukocyte Differentiation in BAL Cells
Cytocentrifuge slides, precoated with PBS/ 0.5% bovine serum albumin (BSA) (wt/vol) (Boseral 20T; Organon Teknika B.V., Boxtel, The Netherlands), were prepared using 100 μl BAL cell suspension (0.3 x 10^6/ml) with a cytocentrifuge (cytospin 3; Shandon, Zeist, The Netherlands) by centrifugation for 5 min at 550 rpm. Slides were stained with May-Grünwald-Giemsa. Percentage of alveolar macrophages (AM), lymphocytes, and neutrophilic and eosinophilic granulocytes were determined by counting 200 cells on two slides each.

Extracellular Cytokines in BALF
IL-6 (23), IL-8 (24) and MCP-1 were measured in duplicate in unconcentrated bronchial and alveolar fractions with an enzyme-linked immunosorbent assay (IL-6 kit [sensitivity: 1.5 pg/ml] from CLB, Amsterdam, The Netherlands; IL-8 [sensitivity: 30 pg/ml] and MCP-1 kits [sensitivity: 15 ng/ml] from R&D Systems, Minneapolis, MN). Levels were not adjusted for total protein as dilution effects resulting from the BAL procedure were comparable between GO and OB groups (Recovery of BALF was not significantly different between both groups as shown in Figure 1.)

Statistical Analysis
Data were analyzed using SPSS/PC+ software (SPSS Benelux b.v., Gorinchem, The Netherlands). Demographic categorical data were compared with the chi-square test. Total cell and differential cell counts, IL-6, IL-8, and MCP-1 concentrations were compared using the nonparametric Mann-Whitney U test. P values 0.05 were considered significant. The effect of IL-6, IL-8, MCP-1, leukocytes, and differentiation on the risk to develop OB was estimated using multiple logistic regression. All regressions were performed separately, with simultaneous adjustment for age, sex, time after lung transplantation, occurrence of CMV infection, and acute rejection at the moment of BAL. Eosinophilic granulocyte counts were transformed in dichotomous data (absence/ presence of eosinophilic granulocytes in the BALF). IL-6, IL-8, MCP-1, leukocytes, differentiation, and the dichotomous eosinophilic data were compared after logarithmic transformation (25).

RESULTS

Population characteristics (Table 1) were not significantly different between the OB and GO groups with regard to age, sex, diagnosis for lung transplantation, and unilateral or bilateral lung transplantation. There were no significant differences in the prevalence of acute rejection and infections at the time of BAL between the OB and GO groups (Table 2). The time lag between lung

transplantation and the first BAL [median values and (ranges) of GO versus OB] was 42 (20 to 300) d versus 41 (27 to 124) d. In the GO group 76% of the patients and in the OB group 90% of the patients underwent BAL within 2 mo after transplantation. Recovery of BALF (median value GO versus OB, 69% versus 74%) (Figure 1) was not significantly different between the OB and GO groups. The median time of histologic evidence of OB was 198 days after transplantation with a range of 80 to 354 d.

The bronchial fraction of the OB patient group had significantly higher numbers of cells/ml in BALF compared with the GO patient group (0.06 x 10^3/ml) versus 0.04 x 10^3/ml)) (Figure 2). The differentiation of cells in the bronchial fraction showed significantly higher numbers of lymphocytes (9 x10^3/ml) versus 2 x 10^3/ml) and eosinophilic granulocytes (1 x 10^3/ml) versus 0) in the OB group than in the GO group (Table 3). Neutrophilic granulocyte counts tended to be higher in the OB group. In the alveolar fraction, the neutrophilic granulocyte counts were significantly higher in the OB group (19 x 10^3/ml) versus 4 x 10^3/ml)) (Table 3), and the eosinophilic granulocyte counts tended to be higher in the OB group. IL-6 and IL-8 concentrations were significantly higher in the OB group than in the GO group both in bronchial (IL-6: 23 versus 6 pg/ml, IL-8: 744 versus 102 pg/ml) and in alveolar fractions (IL-6: 13 versus 3 pg/ml, IL-8: 110 versus 30 pg/ml) (Figure 3B). This was not the case for MCP-1 concentrations, although there was a tendency of higher levels in the alveolar fraction of the OB group ($p = 0.09$).

The association between the cellular and soluble cytokine markers of BALF with the development of OB after lung transplantation was investigated using multiple logistic regression analyses (Table 4). Higher numbers of total cell, neutrophilic granulocyte and lymphocyte counts and the presence of eosinophilic granulocytes in the bronchial fraction, as well as higher IL-6 and IL-8 concentrations, were associated with an increased risk to develop OB (odds ratios of 3.1 to 22.0). In the alveolar fraction higher numbers of total cell and neutrophilic granulocyte counts and higher IL-6, IL-8, and MCP-1 levels were significantly associated with an increased risk to develop OB (odds ratios of 3.8 to 20.5). When comparing IL-6 in presence or absence of CMV infection in GO and OB no significant differences were found (GO bronchial fraction: $p = 0.99$; alveolar fraction: $p = 0.24$; OB bronchial fraction: $p = 0.740$; alveolar fraction: $p = 0.57$). Similar calculations for IL-8 and MCP-1 also showed no differences between GO and OB.

DISCUSSION

The results reported in this prospective cohort study show that early leukocytosis, neutrophilia, presence of eosinophils, and higher concentrations of IL-6 and IL-8 in BALF are associated with the development of OB after lung transplantation. These parameters were studied in both bronchial and alveolar fraction. Differences between the GO and OB group were most often found for cell types in the bronchial fraction. This finding is in agreement with the fact that the bronchial fraction is assumed to be sampled from the proximal airways (21) and OB is a term restricted to membranous and respiratory bronchioles (3, 4) that are part of the proximal airways.

Leukocytosis, especially the influx of neutrophilic granulocytes and lymphocytes, in relation to OB has been reported by several other investigators (8, 26). DiGiovine and coworkers (10) investigated lung transplant patients at the time when OB was diagnosed and also a year before the diagnosis was set. In addition to cell types in the BALF they also evaluated the role of IL-8. They exclu-

sively investigated the alveolar fraction of the lavage fluid and found a trend toward higher concentrations of neutrophilic granulocytes and IL-8 in the so-called "future OB patients" as compared with those not developing OB. We confirm and extend their findings in that we found significantly higher numbers of neutrophilic granulocytes and concentrations of IL-6 and IL-8 in the alveolar fraction of the BALF of the "future OB patients" compared with those of the GO patients. The highly significant differences of neutrophilic granulocytes and IL-8 between the OB and GO groups in our study are probably a result of the restrictive inclusion of the OB group according to Cooper and coworkers (2) and Yousem and coworkers (3). Therefore, IL-8 and neutrophil activity do not only seem to play a role in active OB, but also in developing OB, probably by acting as a profibrotic agent. DiGiovine and coworkers included patients without histologic evidence of OB as well and thereby probably found only trends toward the same direction. Holland and coworkers (27) reported increased lymphocyte counts in BALF of OB patients, in particular $CD8^+$ lymphocytes. In our study, lymphocyte counts were increased in the bronchial fraction, but not in the alveolar fraction.

To our knowledge, eosinophilia in relation to OB has not yet been reported by others. Riise and coworkers (8) and other investigators (9, 10, 26) evaluated the influx of leukocytes and its differentation in association with BOS. They found no relation between eosinophilic counts and BOS. It is important to mention that eosinophils are only found in some of the patients with OB and cannot be used as a general marker of OB but may reflect the severeness of the inflammatory response in the development of OB. Eosinophilic granulocytes are known to be able to release potent cytotoxic granule products associated with the cellular

TABLE 1
POPULATION CHARACTERISTICS

	GO	OB
Patients	41	19
Age, yr (SD)	41.5 (1.8)	39.3 (3.2)
Sex, M/F	20/21	10/9
Diagnosis		
Emphysema/COPD	25	7
CF	6	6
PPH	6	4
Fibrosis	0	1
Bronchiectasis	3	1
Miscellaneous	1	0
Unilateral/bilateral lung transplantation	6/35	5/14

Definition of abbreviations: CF = cystic fibrosis; PPH = primary pulmonary hypertension; COPD = chronic obstructive disease.

TABLE 2
PREVALENCE OF ACUTE REJECTION, CMV, OR OTHER INFECTIONS AT TIME OF BAL

	GO%	OB%
Acute rejection	34	17
CMV	42	42
Fungi	13	6
Bacterial	21	29
Viral other than CMV	5	0

TABLE 3
CELL DIFFERENTIAL COUNTS* IN BALF AFTER LUNG TRANSPLANTATION

Cell Type	GO	n	OB	n
Bronchial fraction				
AM	29 (0–229)	29	36 (13–233)	12
Lymphocytes	2 (0–41)	29	9 (1–64)†	12
Neutrophils	1 (0–128)	29	9 (0–2,328)‡	12
Eosinophils	0 (0–2)	29	1 (0–14)†	12
Alveolar fraction				
AM	206 (11–534)	37	211 (2–1,001)	19
Lymphocytes	9 (0–59)	37	13 (0–55)	19
Neutrophils	4 (0–77)	37	19 (1–3,265)†	19
Eosinophils	0 (0–9)	37	0 (0–44)‡	19

Definition of abbreviation: AM = alveolar macrophages.
* Values are expressed as median (minimum–maximum), $\times 10^3$/ml.
† Mann-Whitney U test: $p < 0.05$, ‡$p < 0.1$, GO versus OB.

TABLE 4
CELLULAR AND SOLUBLE RISK FACTORS IN BALF FOR DEVELOPMENT OF OB

	Odds Ratio (CI)	
	Bronchial Fraction	Alveolar Fraction
Cells	7.7 (1.7–35.2)*	11.1 (1.1–115.6)*
AM	1.6 (0.2–12.3)	1.1 (0.3–5.1)
Neutrophils	4.5 (1.2–16.6)*	5.5 (1.7–18.4)*
Lymphocytes	11.8 (1.3–101.5)*	3.1 (0.7–13.1)
Eosinophils	22.0 (19.8–24.2)*	3.9 (2.3–5.4)†
IL-6	18.2 (2.8–119.5)*	20.5 (3.2–131.3)*
IL-8	3.1 (1.2–8.2)*	3.8 (1.3–11.5)*
MCP-1	3.7 (0.9–14.0)†	3.9 (0.3–55.5)*

Definition of abbreviation: CI = confidence interval.
Significant difference between groups: *$p < 0.05$, †$p < 0.1$.

damage seen in a variety of inflammatory diseases. It is unclear whether eosinophilic granulocytes are actively involved in the development of OB or merely present because of more pronouced activation of and attraction by other cells, for example, epithelial cells. We hypothesize that the presence of eosinophilic granulocytes in the bronchial fraction of the BAL is an early finding in some of the patients who develop OB. The specificity and association with severeness of OB remain to be determined.

To our knowledge, we are the first to report increased IL-6 concentrations in BALF of "future OB patients". Magnan and coworkers (28) reported data on *in vitro* cytokine production by alveolar macrophages isolated from OB patients, showing that the production of IL-6 was not elevated in OB patients, in contrast with the production of TGF-β. They suggested that during OB tissue repair dominated over tissue injury. In our study, patients were monitored shortly after their lung transplantation, with a median value of 42 and 41 d in the GO and OB groups respectively. None of the patients was at that moment classified as having OB. As IL-6 is associated with tissue injury, we hypothesize that patients with elevated IL-6 levels in BALF are the patients at high risk for developing OB and other pathology. Our hypothesis is supported by the finding that in Magnan's study IL-6 concentrations are elevated during acute rejection accompanied by low TGF-β levels. In this study TGF-β was first secreted when IL-6 returned to normal values (28). So IL-6 seems to play an important role in the early stages of developing OB.
Humbert and coworkers also showed elevated IL-6 production in BALF of patients with CMV pneumonia after lung transplantation and to a lesser extent in allograft rejection, suggesting a possible role in OB (13). Because in our study CMV infections were equally divided between the GO and OB groups and because no significant difference was found between GO and OB in IL-6 and other cytokines in the presence or absence of CMV infection, IL-6 seems to play a role in the inflammatory mechanisms of early development of OB.
The elevated levels of the proinflammatory cytokine IL-6, IL-8, MCP-1, chemoattractants, and numbers of leukocytes, (e.g., lymphocytes, neutrophilic and eosinophilic granulocytes) in BALF of patients developing OB after lung transplantation support the indication that OB is caused by an immunologically mediated injury directed against epithelial cells (29). Based upon the study by Borger and coworkers, we hypothesize that the epithelial cell itself may play an important role in the release of IL-6, IL-8, and MCP-1 possibly in part induced by immunosuppressive agents commonly used in lung transplantation (14).
The value of MCP-1 in the pathogenesis of OB has yet to be determined. In our study, a tendency toward higher concentrations in OB was found, together with a significant odds ratio of 3.9 in the alveolar fraction, suggesting additional involvement in the early proces of tissue remodeling. This suggestion is confirmed by preliminary data of Belperio and coworkers (30) who showed a direct relationship between mononuclear cell recruitment and elevation of MCP-1 levels together with CCR2 messenger RNA, which is its major receptor.

The early recognition of the patients at risk to develop OB suggests the possible intervention of this process by anti-inflammatory or more specific medication such as mofetil, FK506, or rapamycine as an inhibitor of cytokine-driven lymphocyte proliferation. Early adjustment of the therapeutic regimen of patients according to their BALF cell count and cytokines profile may contribute to better prevention of OB, but further studies are needed to attain a better defined risk stratification and to gain more

insight into the underlying mechanisms contributing to the development of OB.

We conclude that monitoring total and differential cell counts, IL-6, IL-8, and MCP-1 in BALF within 2 mo after lung transplantation in addition to the TBB pathology will contribute to a better identification and management of groups of patients at risk to develop OB within a year.

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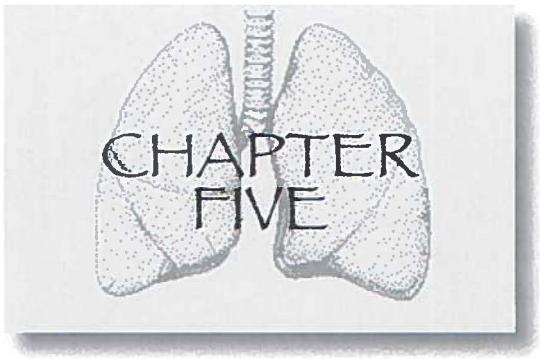
REFERENCES

1. Hosenpud, J. D., L. E. Bennett, B. M. Keck, B. Fiol, and R. J. Novick. 1997. The registry of the international society for heart and lung transplantation: fourteenth official report-1997. J. Heart Lung Transplant. 16:691-712.
2. Cooper, J. D., M. E. Billingham, T. Egan, M. I. Hertz, T. L. Higenbottam, J. Lynch, J. Mauer, I. Paradis, G. A. Patterson, C. Smith, E. P. Trulock, C. Vreim, and S. Yousem. 1993. A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. J. Heart Lung Transplant. 12:713-716.
3. Yousem, S. A., G. J. Berry, P. T. Cagle, D. Chamberlain, A. N. Husain, R. H. Hruban, A. Marchevsky, N. P. Ohori, J. Ritter, S. S. MRCPath, and H. D. Tazelaar. 1996. Revision of the 1990 Working Formulation for the Classification of Pulmonary Allograft Rejection: Lung Rejection Study Group. J. Heart Lung Transplant. 15:1-15.
4. Kramer, M. R., C. Stoehr, J. L. Whang, G. J. Berry, R. Sibley, S. E. Marshall, G. M. Patterson, V. A. Starnes, and J. Theodore. 1993. The diagnosis of obliterative bronchiolitis after heart-lung and lung transplantation: low yield of transbronchial lung biopsy. J. Heart Lung Transplant. 12:675-681.
5. Guilinger, R. A., I. L. Paradis, J. H. Dauber, S. A. Yousem, P. A. Williams, R. J. Keenan, and B. P. Griffith. 1995. The importance of bronchoscopy with transbronchial biopsy and bronchoalveolar lavage in the management of lung transplant recipients. Am. J. Respir. Crit. Care Med. 152:2037-2043.
6. Yousem, S. A., I. Paradis, and B. P. Griffith. 1994. Can transbronchial biopsy aid in the diagnosis of bronchiolitis obliterans in lung transplant recipients? Transplantation 57:151-152.
7. Shennib, H., A. G. L. Lee, C. Serrick, and A. Giaid. 1996. Altered nonspecific lymphocyte cytotoxicity in bronchoalveolar lavage of lung transplant recipients. Can it be useful in monitoring rejection or infection? Transplantation 62:1262-1267.
8. Riise, G. C., A. Williams, C. Kjellstrom, H. Schersten, B. A. Andersson, and F. J. Kelly. 1998. Bronchiolitis obliterans syndrome in lung transplant recipients is associated with increased neutrophil activity and decreased anti-oxidant status in the lung. Eur. Respir. J. 12:82-88.
9. Ward, C., G. I. Snell, L. Zheng, B. Orsida, H. Whitford, T. J. Williams, and E. H. Walthers. 1998. Endobronchial biopsy and bronchoalveolar lavage in stable lung transplant recipients and chronic rejection. Am. J. Respir. Crit. Care Med. 158:84-91.
10. DiGiovine, B., J. P. Lynch, F. J. Martinez, A. Flint, R. I. Whyte, M. D. Ianettoni, D. A. Arenberg, M. D. Burdick, M. C. Glass, C. A. Wilke, S. B. Morris, S. L. Kunkel, and R. M. Strieter. 1996. Bronchoalveolar lavage neutrophilia is associated with

obliterative bronchiolitis after lung transplantation. Role of IL-8. J. Immunol. 157:4194-4202.
11. Zheng, L., E. H. Walters, C. Ward, N. Wang, B. Orsida, H. Whitford, T. J. Williams, T. Kotsimbos, and G. I. Snell. 2000. Airway neutrophilia in stable and bronchiolitis obliterans syndrome patients following lung transplantation. Thorax 55:53-59.
12.Tiroke , A. H., B. Bewig, and A. Haverich. 1999. Bronchoalveolar lavage in lung transplantation. State of the Art. Clin. Transplantation 13:131-157.
13. Humbert, M., R. M. Delattre, S. Fattal, B. Rain, J. Cerrrina, P. Dartevelle, G. Simonneau, P. Duroux, P. Galanaud, and D. Emilie. 1993. Situ production of interleukin-6 within human lung allografts displaying rejection or cytomegalovirus pneumonia. Transplantation 56:623-627.
14. Borger P, Kaufman HF Timmerman JA Scholma J van den Berg JW Koeter GH. 2000. Cyclosporine, FK506, mycophenolate mofetil, and prednisolone differentially modulate cytokine gene expression in human airway-derived epithelial cells. Transplantation 69(7):1408-1413
15. Car, B. D., F. Meloni, M. Luisetti, G. Semenzato, G. Gialdroni-Grassi, and A. Walz. 1994. Elevated IL-8 and MCP-1 in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. Am. J. Respir. Crit. Care Med. 149:655-659.
16. Cooper, J. D. 1990. The evolution of techniques and indications for lung transplantation. Ann. Surg. 212:249-255.
17. Otulana, B. A., T. Higenbottam, L. Ferrari, J. Scott, G. Igboaka, and J. Wallwork. 1990. The use of home spirometry in detecting acute lung rejection and infection following heart-lung transplantation. Chest 97:353-357.
18. Van der Giessen, M., A. P. Van den Berg, W. Van der Bij, S. Postma, W. J. Van Son, and T. H. The. 1990. Quantitative measurement of CMV-specific IgG- and IgM-antibodies in relation to CMV antigenemia and disease activity in kidney recipients with an active CMV infection. Clin. Exp. Immunol. 80:56-61.
19. Van der Bij, W., R. Torensma, W. J. Van Son, J. Anema, J. Schirm, A. M. Tegzess, and T. H. The. 1988. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leucocytes. J. Med. Virol. 25:179-188.
20. Oosterhoff, Y., H. C. Hoogsteden, B. Rutgers, H. F. Kauffman, and D. S. Postma. 1995. Lymphocyte and macrophage activation in bronchoalveolar lavage fluid in nocturnal asthma. Am. J. Respir. Crit. Care Med. 151:75-81.
21. Kelly, C. A., C. J. Kotre, C. Ward, D. J. Hendrick, and E. H. Walthers. 1987. Anatomical distribution of bronchoalveolar lavage fluid as assessed by digital subtraction radiography. Thorax 42: 624-628.
22. Rutgers, S. R., W. Timens, H. F. Kauffman, T. W. van der Mark, G. H. Koeter, and D. S. Postma. 2000. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. Eur. Respir. J. 15:109-115.
23. Pham, S. M. H., Y. Yoshida, R. Aeba, B. G. Hattler, Y. Iwaki, A. Zeevi, R. L. Hardesty, and B. P. Griffith. 1992. Interleukin-6, a marker of preservation injury in clinical lung transplantation. J. Heart Lung Transplant. 11:1017-1024.
24. Smith, J. A., S. Stewart, M. Roberts, K. McNeil, P. M. Schofield, T. W. Higenbottam, S. A. M. Nashef, S. R. Large, F. C. Wells, and J. Wallwork. 1995. Significance of graft coronary artery disease in heart-lung transplant recipients. Transplant. Proc. 27:2019-2020.
25. Altman, D. G. 1991. Relation between several variables. In D. G. Altman, editor Practical statistics for medical research,1 ed. Chapman & Hall/CRC, New York. 351-358.
26. Clelland, C., T. Higenbottam, S. Steward, B. Otulana, T. Wreghitt, J. Gray, J. Scott, and J. Wallwork. 1993. Bronchoalveolar lavage and transbrochial lung biopsy during acute rejection and infection in heart-lung transplant patients. Studies of cell counts, lymphocyte phenotypes, and expression of HLA-DR and Interleukin-2 receptor. Am. Rev. Respir. Dis. 147:1386-1392.
27. Holland, V. A., P. T. Cagle, P. T. Windsor, G. P. Noon, S. D. Greenberg, and E. C. Lawrence. 1990. Lymphocyte subset populations in bronchiolitis obliterans after heart-lung transplantation. Transplantation 50:955-959.
28. Magnan, A., J.-L. Mege, J.-C. Escallier, J. Brisse, C. Capo, M. Reynaud, P. Thomas, B. Meric, L. Garbe, M. Badier, L. Viard, P. Bongrand, R. Giudicelli, D. Metras, P. Fuentes, D. Vervloet, and M. Noirclerc. 1996. Balance between alveolar macrophage IL-6 and TGF-b in lung-transplant recipients. Am. J. Respir. Crit. Care Med. 153:1431-1436.
29. Paradis, I. 1998. Bronchiolitis Obliterans: pathogenesis, prevention and management. The American Journal of the Medical Sciences 315:161-178.
30. Belperio, J. A., Keane, M. P, Burdick, M. D., Xue, Y. Y., Berlin, A. A., Charo, I. R., Kunkel, S. L., and Strieter, R. M. 2000. Elevated levels of MCP-1 are associated with chronic allograft rejection: American Thoracic Society International Conference, May 2000, Toronto (abstract). Am. J. Respir. Crit. Care Med. 161:A508



Airway cellular response to two different immunosuppressive regimens in lung transplant recipients

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Abstract

Background:
Numerous, potentially more effective, immunosuppressive drugs have recently been introduced into transplantation medicine. We investigated the effects of two different immunosuppressive protocols on broncho-alveolar lavage fluid cellular characteristics in 34 lung transplant recipients who were treated with anti-thymocyte globulin induction therapy, cyclosporine, azathioprine and prednisolone ("Regimen-I"), compared to 17 recipients receiving basiliximab induction, tacrolimus, azathioprine and prednisolone ("Regimen-II").
Methods:
Bronchoalveolar lavages were performed between 15 and 40 days post-transplantation, in a stable clinical condition and absence of acute rejection, cytomegalovirus and/or respiratory tract infection.
Results:
Regimen-II treatment was associated with a significantly lower percentage lavage fluid lymphocytes than with Regimen-I .
The CD4/CD8 ratio was significantly higher with Regimen-II than with Regimen-I: 1.56 (range 0.41-2.16) and 0.33 (0.04-0.95) respectively; $p<0.001$, mainly due to a lower percentage $CD8^+$ cells with Regimen-II: 25% (range 12-51) vs. Regimen-I: 60% (34-77); $p<0.001$. The percentage $CD4^+CD25^+$ cells appeared lower with Regimen-II: 21% (range 10-88) vs. Regimen-I: 50% (range 0-87); $p=0.04$.
Conclusions:
Airway lymphocyte subtypes are affected by the immunosuppressive protocol used. The observed cellular differences may explain the better graft- and overall survival, which are attributed to these new immunosuppressive agents.

INTRODUCTION

Immunosuppressive treatment modalities after lung transplantation (LTX) have changed over the past years. Initially, immunosuppressive regimens mainly consisted of induction therapy with ATG (anti-thymocyte globulin) and maintenance therapy with cyclosporine (CsA), azathioprine (AZA) and prednisolone. Newer immunosuppressive drugs like OKT3, daclizumab, basiliximab, tacrolimus (Tac), sirolimus and mycophenolate mofetil (MMF) have recently become available [1-7]. Their introduction in the management of LTX has been supported by positive effects in incidental cases when applied as rescue therapy for allograft rejection, and in small trials showing a favourable outcome with respect to graft function [8-11].

The established and the novel immunosuppressive strategies have a widespread variety in routes of action to prevent allograft rejection, and therefore they might differently affect the airway inflammatory cell population. Especially lymphocyte subtypes have been shown to change considerably after LTX, predominantly as a consequence of immunosuppressive therapy [12]. The effect of the new regimens on cellular populations in the airways of the lung allograft is not known. Evaluation of these cells by using bronchoalveolar lavage fluid (BALF) is feasible, and used to gain more insight into the 'normal' airway changes after LTX as well as into airway changes occurring during both acute and chronic allograft rejection [12-17]. Therefore we set out to study the BALF differential cell population and lymphocyte subtypes in two different immunosuppressive regimens used in our hospital after LTX.

METHODS

Design and subjects

We retrospectively studied the BALF cellular compounds in LTX patients using two different immunosuppressive regimens. Regimen-I was used between 1990-2001 in our LTX-program. Regimen-II from 2001 and onwards (see table 1 for the detailed constituents of the regimens). We included all LTX patients in whom a BAL was performed within the first 40 days after LTX. Exclusion criteria were an acute rejection (AR) episode at time of BAL (as proven by histopathological examination of transbronchial biopsies taken during the same procedure, according to the ISHLT guidelines [18]) and/or a systemic or respiratory tract infection (including active cytomegalovirus (CMV) infection) at time of BAL. LTX procedures were performed according to established techniques [19].

Bronchoalveolar lavage and cell isolation

BAL was performed as described previously [20]. The Medical Ethics Committee approved this protocol. The first 20ml portion was investigated for microbiology. The second 20ml was isolated as bronchial fraction (BF) and three 50ml fractions were pooled for the alveolar fraction (AF). BF and AF were immediately placed on ice (4°C) and further processed for leukocyte differentiation and flowcytometric analysis [12].

Diagnostic protocol

Obtaining positive cultures from the first BALF sample provided a diagnosis of fungal-, viral or bacterial infection. CMV infection was assessed by CMV-antigenemia and CMV serology, as described previously [21,22]. Diagnosis of bronchiolitis obliterans syndrome (BOS) was based on the classification and grading as formulated by the International Society of Heart and Lung Transplantation [23]. Follow up of flow-volume measurements were performed according to established techniques and at a regular basis as described previously [24].

Regimen I	Regimen II
Methylprednisolone, 500mg before reperfusion of each lung and 3x125mg in the first 24hrs post-LTX	Methylprednisolone, 500mg before reperfusion of each lung and 3x125mg in the first 24hrs post-LTX
rATG (anti-thymocyte globulin, Merieux) three doses of 3 mg/kg the first ten days post LTX	Basiliximab (Simulect®), 20 mg on day 0 and 4 post LTX.
Cyclosporine (Neoral®) (serum trough levels: 400 mg/L post-LTX, tapered in 3 weeks to 150 mg/L)	Tacrolimus (Prograft®) (serum trough levels: 20 mg/L the first three weeks post-LTX, 15 mg/L between 3 weeks and 3 months and 10-12 mg/L thereafter)
Azathioprine (2 mg/kg/day)	Azathioprine (2 mg/kg/day)
Prednisolone (0.1-0.2 mg/kg/day)	Prednisolone (0.1-0.2 mg/kg/day)
Co-trimoxazole 980mg on alternate days	Co-trimoxazole 980mg on alternate days and Ganciclovir 3x1000mg/day (the first 3 months post-LTX)

TABLE 1.
Description of the immunosuppressive protocol used for both groups.

Flowcytometric analysis
Lymphocyte subtypes were measured using direct immunofluorescence with monoclonal antibodies as described previously [12]. The following antibodies were used: $CD3^+/CD4^+$, $CD3^+/CD8^+$ (IQ-Products, The Netherlands), $CD4^+/CD25^+$ (Beckton Dickinson, USA). Labelled cells were analysed using a FACS-Calibur (Beckton Dickinson, USA).

Statistical Analysis
Data were analysed using SPSS/PC^+ software (SPSS Benelux, Gorinchem, The Netherlands). Distributions of cell counts, lymphocyte subtypes and cytokine levels were compared between groups using the non-parametrical Mann-Whitney-U test. Chi-square testing was used for calculating differences between in- and excluded patients in the two regimens, between pre-LTX diagnosis, type of LTX and gender. $P< 0.05$ was considered significant. Results are presented as median (range) unless mentioned otherwise.

RESULTS

Patients
We recruited 78 LTX recipients in the Regimen-I group and 25 in the Regimen-II group. In the Regimen-I group 30 patients were excluded because of acute rejection (AR) at time of BAL, and 14 because of a concurrent respiratory tract infection. Of the Regimen-II recipients, 7 patients with AR were excluded (no significant diffe-

	Regimen-I	Regimen-II	
n	34	17	
Age, Years*	43.1 (9.3)	45.1 (11.1)	n.s.
Gender, F/M	16/18	8/9	n.s.#
Pre-LTX diagnosis			
COPD-AT	12	4	n.s.#
COPD	6	5	
CF	6	5	
PF	6	2	
P/SPH	3	1	
re-LTX	1	0	
LTX-type			
Bi-lateral	28	10	n.s.#
Right-lung	4	4	
Left-lung	1	3	
Heart-Lung	1	0	
Time after LTX, days*	27 (6.9)	25 (7.1)	n.s.
Serum trough levels			
Cyclosporine, mg/L*	243.6 (60.4)	-	
Tacrolimus, mg/L*	-	17.3 (4.0)	

TABLE 2.
*Patient characteristics. COPD-AT: α_1-antitrypsin deficiency; COPD: chronic obstructive pulmonary disease; CF: cystic fibrosis; PF: pulmonary fibrosis; P/SPH: primary or secondary pulmonary hypertension; *mean (SD); # Pearson chi-square; n.s.: not significantly different between both groups.*

	Regimen I		Regimen II	
	BF	AF	BF	AF
Recovery, %	35 (3-71)	66 (31-93)	49 (15-64)	60 (8-80)
Total cell, x 10^4	19 (2-642)	21 (5-64)	22 (1-353)	17 (0-63)
Macrophages, %	92 (66-98)	94 (74-99)	96 (32-99)	96 (86-99)
Neutrophils, %	2 (1-30)	2 (0-12)	2 (1-66)	3 (0-9)
Lymphocytes, %	3 (1-9)	4 (0-22)	2 (0-66)	2 (0-7)*
Eosinophils, %	0 (0-7)	0 (0-1)	0 (0-7)	1 (0-4)

TABLE 3.
*Bronchoalveolar lavage fluid total and differential cell characteristics for both regimen groups. BF: bronchial fraction; AF: alveolar fraction (see methods section for explanation). *p=0.034 for Regimen-I compared to Regimen-II.*

rence in prevalence when compared to the number of excluded patients in Regimen-I) and 1 patient with a respiratory tract infection ($p<0.05$ when compared to the number of excluded patients of Regimen-I). In total, 34 out of 78 patients in Regimen-I (44%) and 17 out of 25 patients in Regimen-II (68%) were included for further analysis in this study. Patient characteristics showed no significant differences in age, gender, pre-LTX diagnosis, type of LTX and the time after LTX at which the BAL was performed

(see table 2 for detailed patient characteristics).

Total and differential cell count
There was a significantly lower lymphocyte percentage in the BAL alveolar fraction (AF, see methods section for explanation) in the Regimen-II group than in the Regimen-I group (2% (0-7) vs. 4% (0-22); p=0.034). The total and other differential cell counts for both the BF and AF showed no differences between both regimens (table 3).

Lymphocyte subtypes (alveolar fraction only)
All evaluated lymphocyte subtypes differed significantly between the two regimens (figure 1). $CD3^+$ T-cells were significantly lower with Regimen-II than Regimen-I (79% (47-88) vs. 90% (63-98); p=0.009). $CD4^+$ cells were significantly higher (34% (18-54) vs. 21% (3-41); p=0.001) and $CD8^+$ cells significantly lower (25% (12-51) vs. 60% (34-77); p<0.001) with Regimen-II than Regimen-I, resulting in a much higher CD4/CD8 ratio for regimen II when compared to Regimen-I (1.56 (0.41-2.16) vs. 0.33 (0.04-0.95) p<0.001). Finally, also the percentage of $CD4^+CD25^+$ cells was significantly lower for Regimen-II (21% (10-88) vs. 50% (0-87); p=0.04).

DISCUSSION

Two different immunosuppressive protocols used in patients with LTX were compared in order to obtain more insight into the local airway cell types present early after LTX. We show that there exist remarkable differences in the lymphocyte population of the airways between a regimen consisting of ATG/CsA/AZA/prednisolone and basiliximab/Tac/AZA/prednisolone.

The change of our protocol in 2001 from ATG/CsA/AZA/prednisolone ('Regimen-I') to basiliximab/Tac/AZA/prednisolone ('Regimen-II') was based upon new insights in immunosuppressive therapies that have become available for solid organ transplant recipients. By changing the protocol we eventually tried to achieve a better outcome with respect to overall survival, graft function (freedom of BOS), infectious complications, renal function and post LTX malignancies [1-11].

We used the BAL procedure to evaluate the cellular population of the airways in our patients. BALF examination after LTX is an established diagnostic tool used for microbiological surveillance [25]. BALF is also used to get insight into the "normal" airways changes after LTX, as well as airway changes during AR and bronchiolitis obliterans (BO) [12-17]. We analysed both the bronchial fraction (BF), which is assumed to represent the proximal airways, and the alveolar fraction (AF), reflecting the peripheral airways [19,26]. Total and differential cell counts were found to be comparable in both fractions between the two regimens, suggesting that both the proximal and more peripheral airways are affected in a similar way (table 3).

Intriguingly, the "new" induction therapy using basiliximab (the chimeric interleukin-2 receptor monoclonal antibody) instead of ATG, and a maintenance regimen containing Tac instead of CsA besides AZA and prednisolone, results in a totally different pattern of lymphocyte subtypes in the airways with a much lower percentage of $CD8^+$ and $CD4^+CD25^+$ lymphocytes as major outcome.

It is questionable which component of the regimen used changed the BALF lymphocyte profile. Both CsA and Tac seem to be possible candidates. Both drugs bind to proteins in the cytoplasm to form complexes (CsA binds to cyclophilin and Tac binds to FK-binding proteins), which in turn inhibit the phosphatase activity of calcineurin.

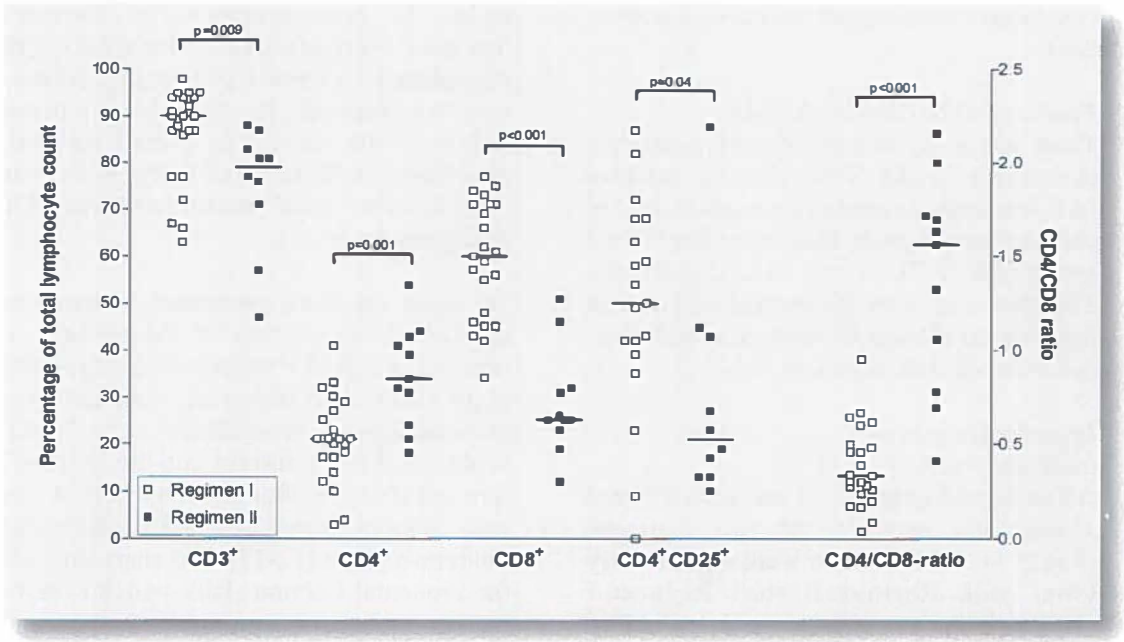

FIGURE 1.

Bronchoalveolar lavage fluid lymphocyte subtype analysis collected within the first 40 days after LTX for Regimen-I (□ n=23): anti-thymocyte globulin induction therapy, maintenance therapy with cyclosporine/azathioprine/prednisolone and Regimen-II (■ n=11*): basiliximab induction, maintenance therapy with tacrolimus/azathioprine/prednisolone. *: number of bronchoalveolar lavage samples suitable for flowcytometric analysis (some could not be performed due to low numbers of lymphocytes obtained and hence inaccurate counts).*

The latter is an important limiting step in the activation of T-cells. By this mode of action inhibition of interleukin-2 (IL-2) production is achieved, resulting in a decreased $CD8^+$ cell population [27-31]. IL-2 is the main cytokine produced by activated $CD4^+$ lymphocytes and is responsible for the growth and proliferation of activated T-cells, thereby playing a vital role in the immune system's rejection response to transplanted organs [32].

Only a few studies have assessed whether differences in lymphocyte subtypes exist between treatment with CsA or Tac after solid organ transplantation. In peripheral blood samples of renal transplant recipients, a regimen consisting of CsA/MMF vs. Tac/MMF only showed small, yet significant differences for $CD3^+$, $CD8^+$ and $CD3^+CD25^+$ lymphocytes [33]. In a rat-heart transplant model, heart tissue flowcytometric analysis showed identical $CD4^+$ and $CD8^+$ levels with both the CsA and Tac regimen [34]. Thus based upon this limited data available and the mode of action of both drugs, the marked differences observed in our BALF lymphocyte subtypes cannot be solely explained by the use of either CsA or Tac.

Another difference between the two regimens is the induction therapy used. ATG reduces the total number of effector T-lymphocytes and blocks their function by direct antigen/antibody interactions [35], whereas basiliximab specifically binds the alpha subunit of the IL-2 receptor of activated T-lymphocytes [6]. By this way of action, basiliximab is potentially capable of reducing the relative number of circulating

$CD8^+$ T-cells. Through competitive antagonism of IL-2, approximately 90% of the available IL-2 receptors on T-lymphocytes have been shown to be complexed and inactivated by basiliximab [6,36].
After induction therapy with basiliximab in renal transplant patients, no $CD25^+$ T-cells were detectable in peripheral blood for 61 days (range 25-93 days). Thus, a lower number of $CD4^+CD25^+$ cells observed in BALF may be explained by the absence of $CD4^+CD25^+$ in peripheral blood by the action of basiliximab [37]. The fact that there still is a low number of $CD4^+CD25^+$ cells in the BALF, can be attributed to donor lymphocytes still present early after LTX. These donor cells are thought to be replaced within a few months post LTX by recipient cells [38-39]. The exact role of the $CD4^+CD25^+$ T-cells still remains speculative. There has recently been renewed interest in the $CD4^+CD25^+$ T-cell population as potent regulatory T cells. It has been shown that a subset of the $CD4^+CD25^+$ T-cell population, the $CD4^+CD25^{high}$ lymphocytes, plays a critical role in the induction of graft tolerance an acceptance after the first months after LTX [40-43]. Unfortunately, the still low numbers of lymphocytes did not allow us to assess whether this specific lymphocyte subset was changed with the new regimen. This requires further study.

Despite remaining controversies with respect to the use of induction therapy after solid organ transplantation [5], we choose to use a protocol containing an induction agent. While ATG, used as induction therapy, has been proven to reduce the number of AR episodes after LTX [35], clinical trails with basiliximab after LTX have yet not been published. However, similar reduction in AR episodes after LTX have recently been published by using either daclizumab (a monoclonal antibody to the α-subunit of the IL-2 receptor on T-cells, with a comparable mode of action as basiliximab), ATG or OKT3 [5]. It also has been reported that basiliximab effectively reduced the number of AR episodes shown in renal transplant recipients [6,37].

As suggested in the literature, the utilisation of the "newer" immunosuppressive drugs will possibly result in a better graft- and overall survival after LTX. However, this has yet not been proven in a randomized trial with sufficient follow up [4,5,8-11].
The BALF lymphocyte profile of Regimen-I suggests the presence of an aggressive allograft response (high $CD8^+$ and high $CD4^+CD25^+$ T-cell population) in the early postoperative phase [16]. The high percentage $CD8^+$ T-cells, when using ATG, has been described previously [44-45]. But the mode of action remains unclear. ATG reduces the total number of $CD3^+$ cells in the peripheral blood after administration. But after ATG therapy, the $CD8^+$ T-cells already have the ability to regenerate despite still depleted $CD4^+$ cells. This may occur due to the loss of control by the depleted $CD4^+$ T-cells and subsequent proliferation of the cytotoxic $CD8^+$ cell population [44-46]. In contrast, with a more specific inhibition by using basiliximab, the controlling $CD4^+$ T-cells cells remain present and thus are able to keep control over the cytotoxic $CD8^+$ T-cells. The Regimen-II lymphocyte population supports this theory, by the presence of a lower lymphocyte percentage, a high CD4/CD8 ratio and a lower $CD4^+CD25^+$ percentage, which likely reflects improved local airway conditions for graft survival. Of importance, the Regimen-II BALF lymphocyte population is nearly identical to BALF characteristics of healthy subjects [47-49].
The Regimen-II patient group did not suffer from over-immunosuppression, as shown by a similar rate of AR episodes at the time of BAL and a lower number of patients with a respiratory tract infection at the time of BAL. A randomised trial with sufficient

long term follow up of complications (AR, BO, respiratory tract infections, post transplant lymphoproliferative disease, and mortality) after LTX seems necessary to show if the observed local cellular improvement in the airways will be reflected in a better clinical outcome.

In conclusion, we were able to show a marked difference in the airway lymphocyte population between two different immunosuppressive protocols used in the management of LTX patients. The protocol containing basiliximab/Tac/AZA showed a favourable lymphocyte subset with respect to immunocompetence, as is best shown by higher percentage of $CD4^+$ and a lower percentage of $CD8^+$ lymphocytes (resulting in a much higher CD4/CD8 ratio) and a much lower $CD4^+CD25^+$ percentage early post-LTX. The observed cellular differences may explain the better graft- and overall survival, which are attributed to these new immunosuppressive agents.

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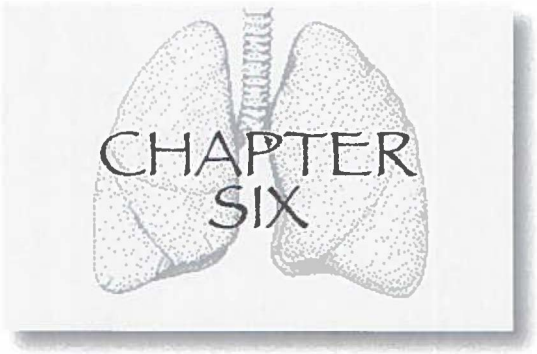
REFERENCES

1. Hausen B, Morris RE. Review of immunosuppression for lung transplantation. Novel drugs, new uses for conventional immunosuppressants, and alternative strategies. Clin Chest Med. 1997;18:353-366.
2. Briffa N, Morris RE. New immunosuppressive regimens in lung transplantation. Eur Respir J. 1997;10:2630-2637.
3. van den Berg JW, Postma DS, Koëter GH, van der Bij W. New immunosuppressive drugs and lung transplantation: last or least? Thorax. 1999;54:550-553.
4. Reichart B, Gulbins H, Meiser BM, Kur F, Briegel J, Reichenspurner H. Improved results after heart-lung transplantation: a 17-year experience. Transplantation. 2003;75:127-132.
5. Brock MV, Borja MC, Ferber L, Orens JB, Anzcek RA, Krishnan J, Yang SC, Conte JV. Induction therapy in lung transplantation: a prospective, controlled clinical trial comparing OKT3, anti-thymocyte globulin, and daclizumab. J Heart Lung Transplant. 2001;20:1282-90.
6. Onrust SV, Wiseman LR. Basiliximab. Drugs. 1999;57:207-13.
7. Sirolimus: Its discovery, Biological properties, and Mechanism of Action. Transplantation Proceedings. 2003;35:7s-14s.
8. Treede H, Klepetko W, Reichenspurner H, Zuckermann A, Meiser B, Birsan T, Wisser W, Reichert B. Tacrolimus versus cyclosporine after lung transplantation: a prospective, open, randomized two-center trial comparing two different immunosuppressive protocols. J Heart Lung Transplant. 2001;20:511-517.
9. Garrity ER Jr, Villanueva J, Bhorade SM, Husain AN, Vigneswaran WT. Low rate of acute lung allograft rejection after the use of daclizumab, an interleukin 2 receptor antibody. Transplantation. 2001;71:773-777.
10. Revell MP, Lewis ME, Llewellyn-Jones CG, Wilson IC, Bonser RS. Conservation of small-airway function by tacrolimus/cyclosporine conversion in the management of bronchiolitis obliterans following lung transplantation. J Heart Lung Transplant. 2000;19:1219-1223.
11. Reichenspurner H, Kur F, Treede H, Meiser BM, Deutsch O, Welz A, Vogelmeier C, Schwaiblmair M, Muller C, Furst H, Briegel J, Reichart B. Optimization of the immunosuppressive protocol after lung transplantation. Transplantation. 1999;68:67-71.
12. Slebos DJ, Scholma J, Boezen HM, Koëter GH, van der Bij W, Postma DS, Kauffman HF. Longitudinal profile of bronchoalveolar lavage cell characteristics in patients with a good outcome after lung transplantation. Am J Respir Crit Care Med 2002;165:501-507.
13. Tiroke AH, Bewig B, Haverich A. Bronchoalve-

olar lavage in lung transplantation. State of the art. Clin Transplant. 1999;13:131-157.
14. Riise GC, Kjellstrom C, Ryd W, Schersten H, Nilsson FN, Martensson G, Andersson BA. Inflammatory cells and activation markers in BAL during acute rejection and infection in lung transplant recipients: a prospective, longitudinal study. Eur Respir J. 1997;10:1742-1746.
15. Zheng L, Walters EH, Ward C, Wang N, Orsida B, Whitford H, Williams TJ, Kotsimbos T, Snell GI. Airway neutrophilia in stable and bronchiolitis obliterans syndrome patients following lung transplantation. Thorax. 2000;55:53-59.
16. Crim C, Keller C, Dunphy C, Maluf H, Ohar J. Flow cytometric analysis of lung lymphocytes in lung transplant recipients. Am J Respir Crit Care Med. 1995;153:1041-1046.
17. Ward C, Snell GI, Zheng L, Orsida B, Whitford H, Williams TJ, Walters EH. Endobronchial biopsy and bronchoalveolar lavage in stable lung transplant recipients and chronic rejection. Am J Respir Crit Care Med. 1998;158:84-91.
18. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Tazelaar HD. Revision of the 1990 Working Formulation for the Classification of Pulmonary Allograft Rejection: Lung Rejection Study Group. J Heart Lung Transplant. 1996;15:1-15.
19. Cooper JD. The evolution of techniques and indications for lung transplantation. Ann Surg. 1990;212:249-255
20. Scholma J, Slebos DJ, Boezen HM, Berg vd JWK, Bij vd W, Boer de W, Koëter GH, Timens W, Kauffman HF, and Postma DS. Eosinophilic granulocytes and interleukin-6 level in bronchoalveolar lavage fluid are associated with the development of obliterative bronchiolitis after lung transplantation. Am J Respir Crit Care Med. 2000;162:2221-2225.
21. Van der Bij W, Torensma R, Van Son WJ, Anema J, Schirm J, Tegzess AM, The TH. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leucocytes. J Med Virol. 1988;25:179-188.
22. Van der Giessen M, van den Berg AP, W. van der Bij, Postma DS, van Son WJ, The TH. Quantitative measurement of CMV-specific IgG- and IgM-antibodies in relation to CMV antigenemia and disease activity in kidney recipients with an active CMV infection. Clin Exp Immunol. 1990;80:56-61.
23. Cooper, J. D., M. E. Billingham, T. Egan, M. I. Hertz, T. L. Higenbottam, J. Lynch, J. Mauer, I. Paradis, G. A. Patterson, C. Smith, E. P. Trulock, C. Vreim, and S. Yousem. A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation. J Heart Lung Transplant. 1993;12:713-716
24. Ouwens JP, van der Mark TW, Koëter GH, de Boer WJ, Grevink RG, van der Bij W. Bronchiolar airflow impairment after lung transplantation: an early and common manifestation. J Heart Lung Transplant. 2002;21:1056-61.
25. Chan CC, Abi-Saleh WJ, Arroliga AC, Stillwell PC, Kirby ThJ, Gordon SM, Petras RE, Metha AC. Diagnostic yield and therapeutic impact of flexible bronchoscopy in lung transplant recipients. J Heart Lung Transplant. 1996;15:196-205.
26. Kelly CA, Kotre CJ, Ward C, Hendrick DJ, Walthers EH. Anatomical distribution of bronchoalveolar lavage fluid as assessed by digital subtraction radiography. Thorax.1987;42:624-628.
27. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature. 1992;357:695-7.
28. Fruman DA, Klee CB, Bierer BE, Burakoff SJ. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc Natl Acad Sci U S A. 1992;89:3686-90.
29. Ho S, Clipstone N, Timmermann L, Northrop J, Graef I, Fiorentino D, Nourse J, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Clin Immunol Immunopathol. 1996;80:S40-5.
30. Matsuda S, Koyasu S. Mechanisms of action of cyclosporine. Immunopharmacology. 2000;47: 119-25.
31. Halloran PF, Kung L, Noujaim J. Calcineurin and the biological effect of cyclosporine and tacrolimus. Transplant Proc. 1998;30:2167-70.
32. Waldmann TA. The IL-2/IL-2 receptor system: a target for rational immune intervention. Immunol Today. 1993;14:264-70.
33. Malinowski K, Ye H, Waltzer WC. Effect of different anti-rejection regimens on the expression of differentiation and activation markers on the surface of host lymphocytes. Transplant Proc. 2000;32: 773-5.
34. Jiang H, Yang X, Soriano RN, Fujimura T, Krishnan K, Kobayashi M. Distinct patterns of cytokine gene suppression by the equivalent effective doses of cyclosporine and tacrolimus in rat heart allografts. Immunobiology. 2000;202:280-92.
35. Palmer SM, Miralles AP, Lawrence CM, Gaynor JW, Davis RD, Tapson VF. Rabbit antithymocyte globulin decreases acute rejection after lung transplantation: results of a randomized, prospective study. Chest. 1999;116:127-33.
36. Boelaars-van Haperen MJ, Baan CC, van Riemsdijk IC, IJzermans JN, Weimar W. Treatment with the chimeric anti-IL-2Ralpha basiliximab affects both the IL-2 and IL-15 signalling pathways after clinical kidney transplantation. Transplant Proc. 2001;33:1007-8.
37. Kahan BD, Rajagopalan PR, Hall M. Reduction of the occurrence of acute cellular rejection among

renal allograft recipients treated with basiliximab, a chimeric anti-interleukin-2-receptor monoclonal antibody. United States Simulect Renal Study Group. Transplantation. 1999;67:276-84.
38. Paradis IL, Marrari M, Zeevi A, Duquesnoy RJ, Griffith BP, Hardesty RL, Dauber JH. HLA phenotype of lung lavage cells following heart-lung transplantation. J Heart Transplant. 1985;4:422-5.
39. Kjellstrom C, Ichimura K, Chen XJ, Riise GC, Collins VP. The origin of alveolar macrophages in the transplanted lung: a longitudinal microsatellite-based study of donor and recipient DNA. Transplantation. 2000;69:1984-6.
40. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev. 2001;182:18-32.
41. Taylor PA, Noelle RJ, Blazar BR. $CD4(^+)CD25(^+)$ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. J Exp Med. 2001;193:1311-8.
42. Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory $CD25(^+)$ T Cells in Human Kidney Transplant Recipients. J Am Soc Nephrol. 2003;14:1643-51.
43. F. Meloni, A. Fietta, L. Ciardelli, I. Mazzucchelli, E. Paschetto, A. Marone Bianco, M. Morosini, A. Cascina, P. Vitulo, E. Pozzi. $CD4^+CD25^{high}CD69^-$ T Cells in Lung Transplant Recipients: Do They Correlate with Tolerance? Am J Respir Crit Care Med 2003;167(7):A939.
44. Wramner L, Robbins DS, Kjellsson B, Mjornstedt L, Olausson M, Brynger H, Soderstrom T. Blood lymphocyte subsets in ATG-treated and allografted rats. Transpl Int. 1990;3:55-8.
45. Muller TF, Grebe SO, Neumann MC, Heymanns J, Radsak K, Sprenger H, Lange H. Persistent long-term changes in lymphocyte subsets induced by polyclonal antibodies. Transplantation. 1997;64: 1432-7.
46. Preville X, Flacher M, LeMauff B, Beauchard S, Davelu P, Tiollier J, Revillard JP. Mechanisms involved in antithymocyte globulin immunosuppressive activity in a nonhuman primate model. Transplantation. 2001;71:460-8.
47. Russi TJ and Crystal RG. Use of bronchoalveolar lavage and airway brushing to investigate the human lung. In Crystal RG and West JB, The Lung, second edition. 1997. Raven Publishers, Philadelphia.
48. Harbeck RJ. Immonuphenotyping of bronchoalveolar lavage lymphocytes. Clin. Diagn. Lab. Immunol. 1998;5:271-77.
49. Mund E, Christensson B, Larsson K, Gronneberg R. Sex dependent differences in physiological ageing in the immune system of lower airways in healthy non-smoking volunteers: study of lymphocyte subsets in bronchoalveolar lavage fluid and blood.Thorax. 2001;56:450-5.



Bronchoalveolar lavage of recurrent sarcoidosis after lung transplantation

CASE-REPORT

J Heart Lung Transplant, In press

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Abstract

End-stage pulmonary disease due to sarcoidosis rarely leads to lung transplantation. Once a patient has undergone lung transplantation, sarcoidosis has been shown to often recur in the lung allograft. In this case report we show, for the first time, the utility of bronchoalveolar lavage fluid in diagnosing recurrence of the sarcoidosis in the transplanted allograft.

INTRODUCTION

Lung transplantation (LTX) is the ultimate therapeutic solution in end-stage lung disease due to various underlying diseases. Besides COPD and idiopathic pulmonary fibrosis, which are limited to the lungs itself, systemic diseases like α_1-antitrypsin deficiency and cystic fibrosis constitute a major indication for LTX [1]. Sarcoidosis is also a systemic disease that may lead to end-stage lung disease but, opposite to other systemic diseases rarely necessitates LTX [2]. However, sarcoidosis is the disease most frequently recurring in the lung allograft after LTX [3-8] despite aggressive immunosuppressive therapy. We here demonstrate for the first time the usefulness of bronchoalveolar lavage fluid (BALF) in diagnosing recurrence of the original sarcoidosis.

CASE

A 44-year-old woman had end-stage lung disease due to sarcoidosis and concurrent secondary pulmonary hypertension for which she was treated with prostacyclin. She underwent an uncomplicated bilateral LTX in June 2001. Eight days post-LTX she was treated once for an acute allograft rejection with 1000 mg methylprednisolone for 3 consecutive days. Nineteen days after LTX she was discharged from the hospital and remained clinically stable. Her immunosuppression included: induction therapy with basiliximab (the chimeric interleukin-2 receptor monoclonal antibody) 20 mg intravenously on days 0 and 4 post LTX. The maintenance regimen consisted of tacrolimus (aimed serum through levels of 20 μg/L during the first three weeks post-LTX, 15 μg/L between 3 weeks and 3 months and 10-12 μg/L thereafter), azathioprine (2 mg/kg/day) and prednisolone (0.2 mg/kg/day). She also received aciclovir and co-trimoxazole prophylaxis. Lung function (both FEV_1 and diffusion capacity) remained perfectly stable during 554 days post-LTX (BOS stage 0; figure 1). Broncho-alveolar lavage (BAL) was performed at regular follow-up visits according to our protocol (i.e. at 3-4 weeks post LTX, and every six months after LTX). BAL was always uneventful. One year after LTX, however, the cellular characteristics of the BALF surprisingly showed an increasing lymphocyte count and elevated CD4/CD8 ratio, suggestive of recurrence of sarcoidosis (table 1). Biopsies taken during the same procedure could not confirm this diagnosis. At the follow-up bronchoscopy performed 490 days post-LTX, the BALF cellular profile was even more characteristic for sarcoidosis (table 1), which on this occasion could be confirmed by the finding of numerous non-caseating granulomas in the transbronchial biopsies (TBB),with negative TBB staining for mycobacteria, and negative BALF cultures and PCR for tuberculosis (figure 2). Until now, at day 554 post LTX, the patient still reached her personal best FEV_1 and no acceleration of the immunosuppressive therapy has been administered. The patient still has no signs of sarcoidosis as shown by lung function and clinic.

DISCUSSION

In this case report we have described the usefulness of BALF in detecting recurrence of sarcoidosis after LTX.

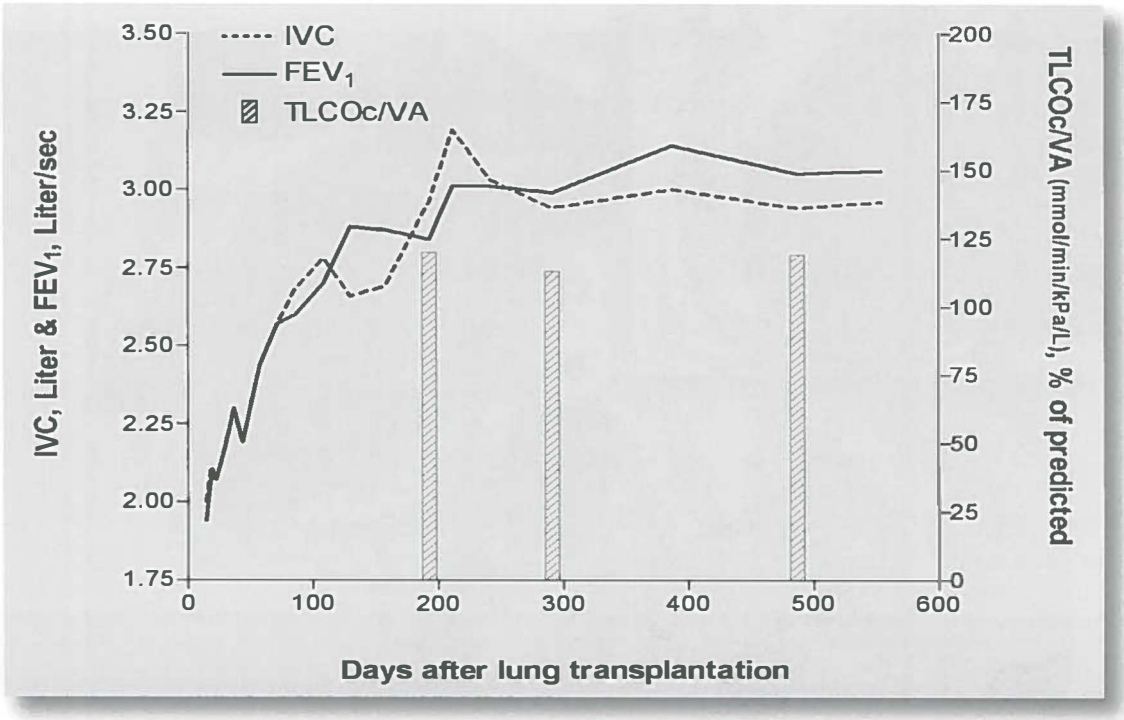
Routinely performed BAL procedures after LTX are useful for microbiological assessment of the allograft [9] and its usefulness for assessment of graft failure is currently under investigation [10,11]. So far no reports are available on using BALF in diagnosing recurrence of diseases like sarcoidosis after LTX. It is now widely accepted that a $CD4^+$ lymphocytosis accompanied by an elevated CD4/CD8 ratio and TBB showing noncaseating granulomas, strongly supports the diagnosis of sarcoidosis in the absence of infectious disease, even though it is not 100% diagnostic [12,13]. We show in this

Days post LTX	17	213	388	490
*Broncho-alveolar lavage**				
Recovery, %	77	65	77	75
Total cell count, x10^4/mL	2.16	2.44	1.87	2.50
Macrophages, %	88	96	79	72
Lymphocytes, %	7	4	20	26
Neutrophils, %	5	0	1	2
Eosinophils, %	0	0	0	0
$CD4^+$ cells, x10^3/mL	NP	NP	3.80	3.81
$CD8^+$ cells, x10^3/mL	NP	NP	0.75	0.51
CD4/CD8 ratio	NP	NP	5.06	7.45
Lavage microbiology	neg.	neg.	neg.	neg.
AR/BO on biopsies	neg.	neg.	neg.	neg.
Serum				
ACE, U/L	13	9	15	14
CRP, mg/L	5	<3	<3	<3

TABLE 1.
*Longitudinal Bronchoalveolar lavage (BAL) and serum characteristics. *BAL is performed by using 3x50 mL pre-warmed phosphate buffered saline as described previously [11]. NP= not performed due to low numbers of lymphocytes obtained and hence inaccurate counts. neg. = negative; AR = acute rejection; OB = bronchiolitis obliterans; ACE = angiotensin converting enzyme; CRP = C-reactive protein.*

case, that also the recurrence of sarcoidosis after LTX can be determined in this way.
Only 1-2% of the LTX recipients reported in the literature have been transplanted because of end-stage lung disease due to sarcoidosis [2,3]. Until now there are only a few series available reporting the recurrence of sarcoidosis in the lung allograft [3-5]. A recent study assessing the prevalence of radiological findings compatible with recurrent underlying disease in general in 1394 LTX recipients showed an overall disease recurrence rate of 1%. In this group, sarcoidosis was the main cause of recurrent disease (nine out of 26 transplants) [3].
It is suggested that acute rejection episodes and bronchiolitis obliterans may occur

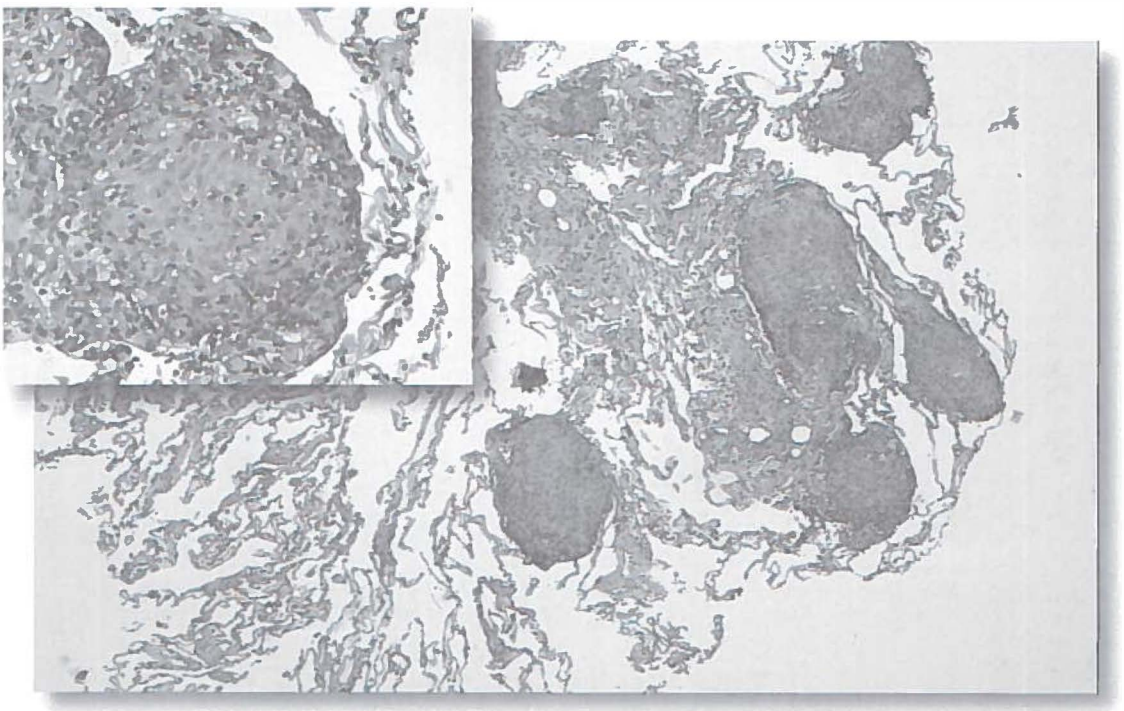
FIGURE 1.
Post lung transplantation lung-functional characteristics. Solid line represents FEV_1; dotted line IVC (inspiratory vital capacity) (both plotted on the left Y-axis). The bars (plotted against the right Y-axis) represent the diffusion capacity as given in percentage of predicted TLCOc/VA (mmol/min/kPa/L).



more frequently in patients with sarcoidosis when compared to non-sarcoidosis LTX recipients [4,7,8]. However, the number of patients (7 in total) studied in these groups is too small to draw firm conclusions. A more recent report (including 12 sarcoidosis patients as recipients) could not confirm this previous suggestion of accelerated allograft failures and also found no difference in survival with respect to sarcoidosis as the underlying disease for LTX [5]. However, as shown in the international registry [1], the better outcome of patients with organ-limited lung diseases as COPD and α_1-antitrypsin deficiency suggests that an underlying systemic disease may indeed influence overall outcome after LTX. We only report one patient, yet she had just one post-LTX episode of acute rejection, with a 100% preserved graft function and normal thoracic X-ray up to day 554 post-LTX, despite the numerous granulomas found on histopathological examination.

Despite the aggressive immunosuppressive regimen after LTX, sarcoidosis appears to recur in our patient. This has been previously reported with a prevalence of 25-80% [3-8]. Aggravation of pulmonary sarcoidosis has also been reported to occur in renal- and liver transplant recipients [14,15]. Thus, despite aggressive inhibition of the T-cell response, sarcoidosis develops in the allograft. In our patient, the cellular profile of the BALF matching with sarcoidosis, is rather unaffected by the immunosuppressive regimen when compared to normal values of BALF cellular characteristics after LTX, where the number of lymphocytes is normally 2-4% instead of the 20-26% found in our patient [11]. This suggests that besides the ability to develop granulomas,

FIGURE 2.
Transbronchial biopsy showing noncaseating granulomas in the lung allograft. (hematoxylin & eosin staining; magnifications: total biopsy 20x, magnified granuloma 100x)



also the $CD4^+$ lymphocytic alveolitis is unaffected by the immunosuppression. It is hard to understand the fact that despite the immunosuppressive treatment, sarcoidosis returns. This suggests that sarcoidosis is not driven by deficiencies in the T-cell reactivity, but may be due to tissue-driven defects. This is also illustrated by a clinical report on the lack of effect of cyclosporine in the treatment of pulmonary sarcoidosis [16].

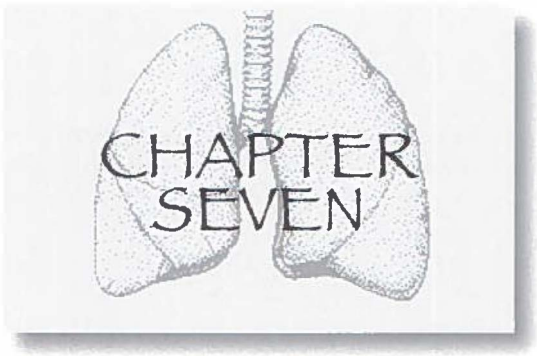
Because of excellent graft function as reflected by near normal lung function values and the absence of clinical signs of sarcoidosis, we did not change the immunosuppressive regimen in our patient. When indications for treating the sarcoidosis will present e.g. progressive graft failure due to the sarcoidosis, the first step in treatment seems to raise the dose of prednisolone up to 0.5 mg/kg/day [13]. Besides this opinion based treatment, there is no current opinion on how to treat these patients who are already on a very aggressive immunosuppressive regimen. Future possibilities may imply novel strategies like anti-TNFα or thalidomide treatment. Because the number of LTX patients with sarcoidosis remains small, and additive therapy is rarely warranted, attempts with these agents will have to be made based upon non-LTX experiences [17,18].

In conclusion, end-stage pulmonary disease due to sarcoidosis rarely leads to lung transplantation. Once transplanted, and despite aggressive immunosuppressive treatment, sarcoidosis is the disease most often recurring in the lung allograft. This recurrence seems, however, to have no impact on survival. In this case report we show for the first time, the utility of BALF examination in diagnosing the recurrence of sarcoidosis in the lung-allograft. Remarkably, the BALF cellular profile in this recurrent sarcoidosis seems unaffected by the aggressive immunosuppressive regimen used.

REFERENCES

1. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, and Novick RJ. The registry of the international society for heart and lung transplantation: seventeenth official report. J Heart Lung Transplant 2000;19:909-931
2. Judson MA. Lung transplantation for pulmonary sarcoidosis. Eur Respir J. 1998;11:738-744
3. Collins J, Hartman MJ, Warner TF, Muller NL, Kazerooni EA, McAdams HP, Slone RM, Parker LA. Frequency and CT findings of recurrent disease after lung transplantation. Radiology. 2001;219: 503-9
4. Johnson BA, Duncan SR, Ohori NP, Paradis IL, Yousem SA, Grgurich WF, Dauber JH, Griffith BP. Recurrence of sarcoidosis in pulmonary allograft recipients. Am Rev Respir Dis. 1993;148:1373-7
5. Walker S, Mikhail G, Banner N, Partridge J, Khaghani A, Burke M, Yacoub M. Medium term results of lung transplantation for end stage pulmonary sarcoidosis. Thorax. 1998;53:281-4
6. Martinez FJ, Orens JB, Deeb M, Brunsting LA, Flint A, Lynch JP 3rd. Recurrence of sarcoidosis following bilateral allogeneic lung transplantation. Chest. 1994;106:1597-9
7. Bjortuft O, Foerster A, Boe J, Geiran O. Single lung transplantation as treatment for end-stage pulmonary sarcoidosis: recurrence of sarcoidosis in two different lung allografts in one patient. J Heart Lung Transplant. 1994;13:24-9
8. Muller C, Briegel J, Haller M, Vogelmeier C, Bittman I, Welz A, Furst H, Dienemann H. Sarcoidosis recurrence following lung transplantation. Transplantation. 1996;61:1117-9
9. Tiroke AH, Bewig B, Haverich A. Bronchoalveolar lavage in lung transplantation. State of the art. Clin Transplant 1999;13:131-157
10. Scholma J, Slebos DJ, Boezen HM, Berg vd JWK, Bij vd W, Boer de W, Koëter GH, Timens W, Kauffman HF, and Postma DS. Eosinophilic granulocytes and interleukin-6 level in bronchoalveolar lavage fluid are associated with the development of obliterative bronchiolitis after lung transplantation. Am J Respir Crit Care Med 2000;162:2221-2225
11. Slebos DJ, Scholma J, Boezen HM, Koëter GH, van der Bij W, Postma DS, Kauffman HF. Longitudinal profile of bronchoalveolar lavage cell characteristics in patients with a good outcome after lung transplantation. Am J Respir Crit Care Med 2002;165:501-507
12. Winterbauer RH, Lammert J, Selland M, Wu R, Corley D, Springmeyer SC. Broncho-alveolar lavage cell populations in the diagnosis of sarcoidosis. Chest. 1993;104:352-61
13. The American Thoracic Society (ATS), The European Respiratory Society (ERS) and World Association of Sarcoidosis and Other Granulomatous

Disorders (WASOG). Statement on sarcoidosis. Am J Respir Crit Care Med 1999;160:736-755
14. Schmidt RJ, Bender FH, Chang WW, Teba L. Sarcoidosis after renal transplantation. Transplantation. 1999;68:1420-3
15. Shibolet O, Kalish Y, Wolf D, Pappo O, Laxer U, Berkman N, Shaham D, Ashur Y, Ilan Y. Exacerbation of pulmonary sarcoidosis after liver transplantation. J Clin Gastroenterol. 2002;35:356-8
16. Wyser CP, van Schalkwyk EM, Alheit B, Bardin PG, Joubert JR. Treatment of progressive pulmonary sarcoidosis with cyclosporin A. A randomized controlled trial. Am J Respir Crit Care Med. 1997;156: 1371-6.
17. Baughman RP, Judson MA, Teirstein AS, Moller DR, Lower EE. Thalidomide for chronic sarcoidosis. Chest. 2002;122:227-32
18. Yee AM, Pochapin MB. Treatment of complicated sarcoidosis with infliximab anti-tumor necrosis factor-alpha therapy. Ann Intern Med. 2001;135: 27-31



Feasibility of sputum induction in lung transplant recipients

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Abstract

Sputum induction (SI) is nowadays applied as a non-invasive and safe method to investigate airway inflammation in pulmonary diseases.
We investigated the feasibility of SI after lung transplantation (LTX), and compared sputum and bronchoalveolar lavage (BAL) cellular characteristics and interleukin-8 (IL-8) levels. Results were also compared to 11 healthy subjects.
SI as performed between 26 and 1947 days after LTX in 19 recipients, was successful in 16 out of 22 attempts (73%). Six patients failed to produce sputum after induction, mostly just post-LTX and with having a lower FEV_1. The success rate in clinically stable patients after the first month post-LTX was 93%. Side effects were absent. Sputum recovery, viability and squamous cell contamination were comparable between LTX patients and healthy subjects. In the LTX group total cell counts, neutrophil percentages and IL-8 levels were much higher in SI than BAL (1.6×10^6/ml, 65.5% and 54.2 ng/ml versus 0.1×10^6/ml, 3.0% and 0.01ng/ml $p<0.001$). Although LTX-neutrophil percentages in SI and BAL correlated properly ($rho=0.72$, $p=0.04$), both techniques are not exchangeable.
We conclude that sputum induction is feasible, well tolerated, and without major side-effects in stable patients after the first month post LTX. Induced sputum may be a useful tool to study inflammatory changes of the airways after LTX, and because of the large quantity of neutrophils sampled, especially for further studies on the pathogenesis of bronchiolitis obliterans.

INTRODUCTION

Chronic pulmonary allograft rejection, or bronchiolitis obliterans (BO), has emerged as the major long-term complication after lung transplantation (LTX) and is associated with increased morbidity and mortality [1,2]. In affected patients, medical therapy results at best in stabilisation of lung function, but has been largely ineffective in restoring lung function [3,4]. Because of this lack of effective therapy, much emphasis is put on identifying early markers of BO in order to elucidate the pathophysiology of the disease and to prevent it from progressing to a full-blown syndrome of severe airway obstruction [5]. To this aim the 'bronchiolitis obliterans syndrome' has been introduced, a classification based upon deterioration in the forced expiratory volume in 1 second (FEV_1) from its baseline value [6]. But airway pathological changes are already manifest even before decline in FEV_1 develops [5]. More invasive procedures like transbronchial biopsies (TBB) are often used in follow-up of LTX patients, and even open lung biopsies are sometimes performed to detect graft failure. However, they all show a low sensitivity to diagnose BO [7-9].

Bronchoalveolar lavage fluid (BALF) analysis serves an important clinical purpose in the detection of microbiological and viral complications after LTX, and is being used to try to assess graft failure, i.e. bronchiolitis obliterans (BO) and acute rejection (AR) [10-14]. It thus has been shown that analysis of BALF compounds reveals some prognostic implications [5,15]. However, the invasiveness and high costs of bronchoscopy make repeated analysis of BALF less suitable for frequent follow-up studies to monitor LTX patients. Sputum induction (SI) is a less invasive and save technique in other diseases [16-18] and might constitute an adequate technique in LTX, since small airways disease corresponding with BO may be associated with or preceded by chronic inflammation of larger airways [19-22].

In the present study, we tested the feasibility of sputum induction in LTX recipients, and compared its cellular composition and level of IL-8 with values in BALF. Finally, we compared results of SI and BALF with values obtained in healthy volunteers.

METHODS

Design & Subjects

Between October 1998 and June 1999, recipients of bilateral lung transplants who were scheduled for a bronchoscopy with BAL were asked to participate in the present study. The bronchoscopies were scheduled for routine surveillance reasons (follow-up after LTX: detection of graft failure), or clinical indication (suspicion of infectious disease or acute rejection). SI took place between 48 and 2 hours prior to the BAL. Eleven healthy non-smoking volunteers (8 males and 3 females) participated as control subjects (age 58 ± 8 years). They did not have a history of pulmonary disease, and had a normal lung function (FEV_1 104% of predicted ±11%). All research methods were approved by the Medical Ethics Committee, and written informed consent was given.

Therapeutic regimen

Immunosuppression included 2-5 doses of antithymocyte globulins (rATG, Merieux, 3 mg/kg) the first 10 days post-LTX. The maintenance regimen consisted of cyclosporin (serum levels: 400 ng/ml post-LTX, tapered in 3 weeks to 150 ng/ml), azathioprine (1-3 mg/kg/day) and prednisolone (0.1-0.2 mg/kg/day). One patient received mycophenolate mofetil (1000 mg twice daily) in conjunction with cyclosporin and prednisolone. All patients received aciclovir and co-trimoxazole prophylaxis.

Sputum induction
SI was performed via inhalation of nebulized concentrations hypertonic saline of 3%, 4% and 5% during 7 min each, generated by an ultrasonic nebulizer (Ultraneb 2000, DeVilbiss, Somerset, Pennsylvania, USA) as described before [23]. The nebulizer was calibrated at an output of 1.5 ml/min and produced particles with a diameter of 4.5 μm. After each concentration of saline, and after thoroughly cleaning of the upper airways, they expectorated the sputum into a sterile container, which was kept on ice and processed within 15 min after induction. The procedure was terminated after the three periods of 7 min or if a drop in FEV_1 of 20% or more from baseline would occur, which did not happen in our study. A SI failure was defined as having produced no sputum.

Sputum processing
In this study the 'whole sample technique' was used [23]. The weight of the sample was determined, and a volume equal to weight of dithiothreitol 0.1% (Sputolysin; Calbiochem, La Jolla, CA, USA) was added. The samples were vortexed, and homogenized in a shaking water bath at 37° C for 15 min. The samples were then vortexed, and filtered through a 48 μm nylon gauze. Total cell count (including squamous cells) was performed in the filtered sample, and viability was checked by trypan blue exclusion. The sample was then centrifuged at 450 x g at 4° C for 10 min. The supernatant was aspirated and stored at -80° C. The cell pellet was resuspended in Bovine Serum Albumin (BSA) buffer (0.5% BSA in Phosphate Buffered Saline (PBS)) to a concentration of 0.3 x 10^6 cells/ml for cytospinning (100 μl of the cell suspension) Two cytospins were stained with May-Grünwald-Giemsa for cell differentials, counting 300 nonsquamous cells in each cytospin. Total cell count was calculated by multiplying the cell percentage with the total (nonsquamous) cell number in sputum, divided by the volume of the sputum sample.

Bronchoscopy and BALF processing
BAL was performed as described before [5]. A flexible bronchoscope (Olympus B1 IT10, Olympus Optical, Tokyo, Japan) was introduced orally or nasally and wedged in a subsegmental bronchus of the middle lobe. BAL was performed by using PBS. A first 20 ml portion was investigated for viruses, bacteria and fungi, a second 20 ml portion was isolated as bronchial fraction (these results were not further analysed for this study) and three 50 ml aliquots were pooled and labelled BAL. Each aliquot was immediately recovered using a negative pressure < 20 mm Hg. The BALF was placed on ice and processed within 10 min after recovery. It was filtered through a 200 μm nylon gauze and recovery and aspect were assessed. The lavage was centrifuged at 400xg at 4° C for 5 min, and supernatants were decanted and stored in Eppendorf cups at -80° C. The cell pellet was resuspended in PBS containing 0.1% Glucose, and once again centrifuged. The supernatant of the second centrifugation was aspirated and the cell pellet was resuspended in PBS containing 0.1% Glucose for total cell count, viability assessment and cytospin spinning. Total cell counts, differentials and IL-8 measurements were performed in a manner identical to SI.

BIOCHEMICAL ASSAY
Interleukin-8 (IL-8) was measured using (h)IL-8 ELISA, detection limit 1pg/ml (Amersham, Little Chalfont, GB).

STATISTICS
Cell characteristics are presented as medians (range). Comparison between differential cell counts (percentage of nonsquamous cells) in sputum and in BAL and between differential cell counts in transplant recipients and healthy volunteers was made

with the Kruskall-Wallis test and the Mann-Whitney U test. Cell variable correlations were analysed using the Spearman's rank correlation coefficient. The Bland and Altman comparison method for assessing agreement between two methods of clinical measurement was used to compare SI and BAL [24]. A p-value <0.05 was considered significant.

TABLE 1.
Patient characteristics.
**: p<0.05 for successful induction vs unsuccessful induction (values expressed as median (min-max)). #: Indication for bronchoscopy, 'P' indicates a routine follow-up bronchoscopy, 'C' means a bronchoscopy on clinical indication. ¶: BOS%: current FEV_1/personal best FEV_1*100%, between parenthesis the complimentary BOS stage (ISHLT guidelines [6]). Abbreviations: M= male, F= female; AT-DEF= α_1-antitrypsin deficiency, CF= Cystic Fibrosis, PF= pulmonary fibrosis, SPH= secondary pulmonary hypertension, BR= bronchiectasis, COPD= chronic obstructive pulmonary disease, LAM= lymphangioleiomyomatosis, AR= acute rejection.*

Patient			Primary diagnosis	Days post LTX	FEV_1(L)	BOS%¶(stage)	Indication#	Findings on bronchoscopy
No	Age	M/F						
Successful induction								
1	52	M	AT-DEF	26	2.71	95.1 (0)	P	*S.Aureus, CMV*
2	33	M	AT-DEF	223	3.14	87.2 (0)	P	-
3	28	F	CF	577	3.00	93.8 (0)	P	-
4	48	M	PF	1947	2.63	78.0 (1)	C	*AR grade 1*
5	26	M	SPH	745	3.18	75.0 (1)	P	-
6	46	F	AT-DEF	197	3.54	100.0 (0)	P	-
7	46	F	AT-DEF	365	3.69	92.3 (0)	P	-
8	55	M	AT-DEF	41	3.36	100.0 (0)	P	*P.Aeruginosa*
9	28	F	CF	95	3.18	86.7 (0)	P	*P.Aeruginosa*
10	28	F	CF	191	3.27	89.1 (0)	P	-
11	29	F	BR	1245	2.81	65.4 (1)	C	-
12	51	M	AT-DEF	564	3.02	65.7 (1)	P	-
13	24	F	SPH	755	2.37	98.8 (0)	P	-
14	44	M	AT-DEF	376	4.05	95.5 (0)	P	-
15	21	M	CF	60	1.38	81.2 (0)	P	*P.Aeruginosa*
16	21	M	CF	193	1.23	72.4 (1)	P	*P.Aeruginosa*
36.2(±12.1)				*294* (26-1947)*	*3.10* (1.23-4.05)*	*86.0 (62.4-100)*		
Unsuccessful induction								
17	50	F	SPH	1643	2.62	77.0 (1)	C	
18	51	F	COPD	572	2.45	92.3 (0)	P	
19	43	M	AT-DEF	21	2.24	100 (0)	P	
20	51	F	COPD	23	1.67	100 (0)	P	
21	40	F	LAM	33	1.37	91 (0)	P	
22	53	F	AT-DEF	21	2.70	100 (0)	P	
48.0 (±5.2)				*28 (21-1643)*	*2.35 (1.37-2.70)*	*93.4 (77-100)*		

RESULTS

Patients, Sputum induction and BAL (table 1.)

Nineteen LTX-recipients participated (see table 1 for patient characteristics). In three patients SI was performed twice, thus 22 SI were evaluated. SI was performed at a median of 294 days after transplantation (range 26-1947 days). SI was 16 times successfully induced (73%), whereas this was

100% in the healthy subjects ($p<0.05$). SI could be performed both early and late after transplantation, but the failure rate in our group of patients was higher just post LTX ($p<0.05$). When evaluating all SI procedures performed after the first month post-LTX the success rate was 15/17: 88%. When only performing SI in clinically stable patients after the first month post-LTX, the success rate was 14/15: 93%. The patients who failed to produce sputum had a significantly lower FEV_1 compared to those who succeeded (median FEV_1 2.35 versus 3.10 L, respectively, $p<0.05$). No differences in age and gender could be found between the groups with successful and unsuccessful procedures. All participants tolerated the procedure well, although many reported the salty taste to be unpleasant. All but three bronchoscopies (and BAL) were performed for routine surveillance; in one patient, who

TABLE 2.
Cellular characteristics and IL-8 concentration in induced sputum and BALF of LTX recipients and healthy subjects. Values are expressed as medians (min-max). SI: sputum induction. BALF: bronchoalveolar lavage fluid. TCC: total cell count.
¶: $p<0.005$ between LTX and healthy subjects;
**: difference between results from SI and BALF within each group.*

	SI		BALF		*SI versus BAL*
LTX	n=16		n=15		*p value**
TCC, $*10^6$/ml	1.6¶	(0.6-13.4)	0.1¶	(0.01-0.42)	*<0.001*
macrophages, %	31.0¶	(7-98)	91	(56-97)	*<0.001*
neutrophils, %	65.5¶	(18-92)	3.0¶	(1-42)	*<0.001*
lymphocytes, %	2	(0-7)	3.0¶	(1-41)	*ns*
eosinophils, %	0	(0-16)	0	(0-7)	*ns*
IL-8, ng/ml	54.23¶	(1.4-116)	0.01¶	(0.003-0.56)	*<0.001*
Healthy	n=11		n=11		
TCC, $*10^6$/ml	1.2	(0.8-3.2)	0.1	(0.03-0.2)	*0.003*
macrophages, %	57	(34-78)	92	(70-93)	*0.003*
neutrophils, %	36	(18-60)	1	(0-3)	*0.003*
lymphocytes, %	3.5	(1-8)	7	(2-26)	*0.01*
eosinophils, %	0	(0-3)	0	(0-1)	*ns*
IL-8, ng/ml	4.6	(3.1-7.7)	0.008	(0-0.06)	*0.003*

underwent SI twice, BAL yielded no recovery.

Sputum data
Median sputum recovery was 3.0 ml (range 0.7-16.9 ml) in the LTX patient group and 5.7 ml in the healthy control subjects (3.9-16.3 ml). Median sputum weight was 3.8 g (1.0-21.8 g) and mean viability was 77% (±15) in the LTX group. The amount of squamous epithelial cells was 6% (0-41%) in the LTX group and 6% (3-14%) in the healthy control subjects.

LTX and Healthy subjects within group analysis
Total cell counts, percentages of neutrophils and levels of IL-8 were significantly higher in SI than in BALF of the LTX patient group (all $p<0.001$). Similar results were found when comparing SI and BAL in the healthy volunteers, with the exception of the percentage of lymphocytes, which was significantly higher in BAL (7% (2-26)) than in SI (3.5% (1-8)) ($p=0.01$; table 2). The neutrophil percentage in BAL and SI in the LTX patients were positively correlated (rho=0.72, $p=0.04$; figure 1). The Bland and Altman comparison between SI and BAL showed, with an exception for the lymphocyte percentage, a constant proportional error between both methods for neutrophils, IL-8 levels and total cell counts (figure 2 A-D) [24].

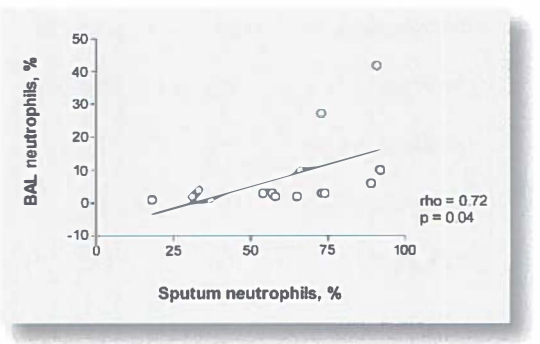
LTX vs. Healthy subjects
Total cell counts, neutrophil percentages and IL-8 levels were significantly elevated in SI of the LTX patient group when compared to healthy volunteers (all $p<0.005$), whereas the percentage of macrophages was lower for the LTX group (table 2). Total cell counts, percentages of neutrophils, and levels of IL-8 were higher in BAL of LTX recipients, whereas lymphocyte percentages were lower than in the BAL of healthy subjects (all $p<0.005$).

DISCUSSION

The present study shows that sputum induction after lung transplantation is feasible, well tolerated and without major side effects in stable patients after the first month post-LTX. SI was performed successfully from as early as 26 days to 1947 days after LTX with an overall success rate of 73%.

The success rate of SI appears to depend both on the time post LTX and the level of FEV_1. SI was not tried in our patient group before 20 days after LTX and unsuccessful SI procedures were most times noted in patients investigated during the first month after LTX and with lower FEV_1. At that time impaired coughing and general weakness, due to the recent thoracic surgery, impaired the patients to produce sputum. Low FEV_1 values shortly after LTX do not signify disease, i.e. rejection or BOS, but reflect rather a normal recovery of lung function after transplantation. Lung function generally continues to improve during at least the first 90 days after LTX [25]. Of importance is that SI was almost every time successful when performed in stable patients after the first month post LTX, encompassing a substantial range of FEV_1 values,

FIGURE 1.
Correlation (Spearman's rho) of neutrophil percentage between sputum induction (SI) and broncho alveolar lavage (BAL).

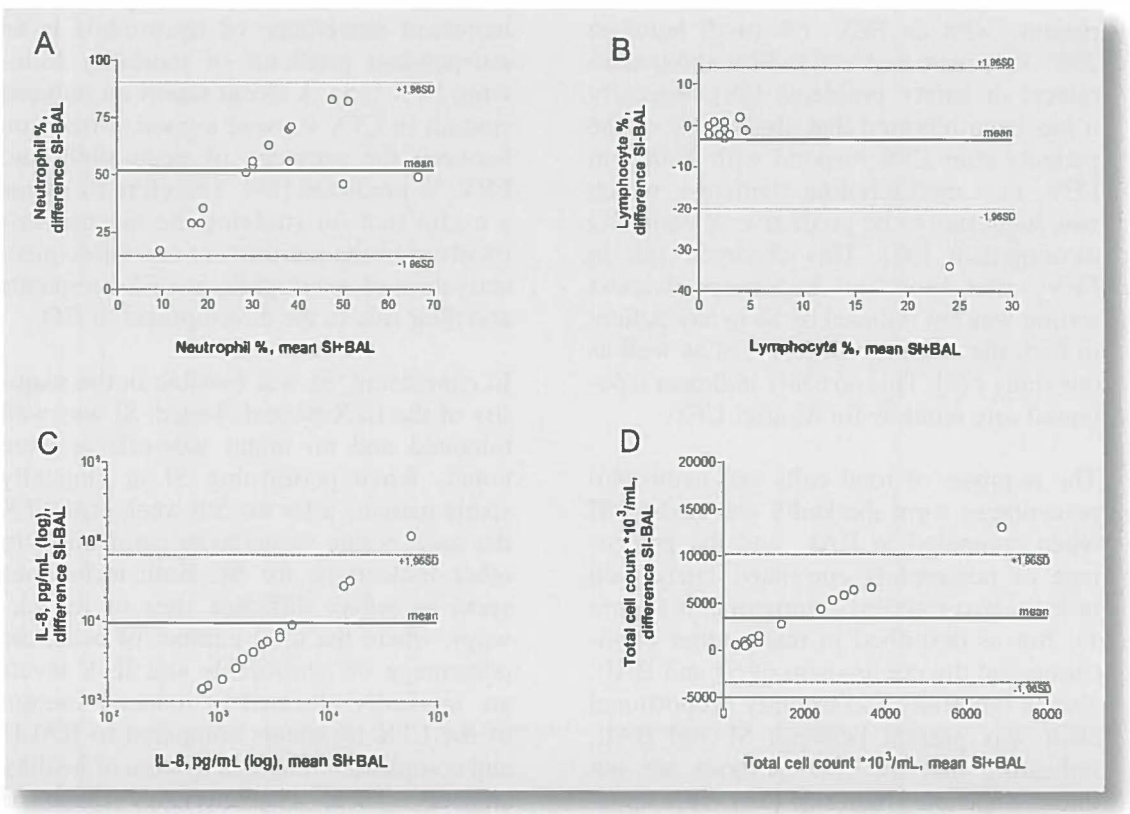


SI was performed before the BAL procedure. It is known that SI can induce neutrophilia in case of repeated SI procedures [26-27]. However, no studies are available regarding the effect of SI on BAL. Theoretically this might have influenced our results. Since different sites of the airways are examined by the two methods, the BAL-neutrophil percentages in the LTX patient group resemble our own LTX-BAL reference values, and we did not notice a difference in neutrophil percentages in BALF that was collected on the different days after SI, this effect seems limited [14, 23].

We have included patients with bilateral LTX only in our study. Thus we cannot comment whether it is to be advised to apply SI in single LTX patients as well. Beech et al showed that SI was similarly tolerated in case of bilateral and single LTX [28]. We did not include single LTX patients as to exclude possible cellular contamination from the underlying diseases present in the acceptor lung. Furthermore Beech et al did even not compare cellular profile between single and bilateral LTX patients in their study, and properly observed that the underlying disease forcing the choice for single

FIGURE 2 (A-D).

Bland and Altman comparisons for assessing agreement between two methods of clinical measurement for A) neutrophil %, B) lymphocyte %, C) IL-8 levels and D) total cell counts between sputum induction (SI) and broncho alveolar lavage (BAL) [24]. Values on the X-axis show the mean of both methods, whereas the Y-axis shows the difference between SI and BAL. Mean (±1.96SD) is given for the difference between SI and BAL. Figures A,C and D show a constant proportional error between both methods.



or bilateral LTX may affect the outcome of SI as well (i.e. emphysema or pulmonary fibrosis). Thus only studies comparing single and bilateral LTX patients with the same type of disease may shed light as to the question whether SI can be used in single LTX patients as well.
SI ha a success rate of 93% in stable patients after the first month post LTX, comparable to reported success rates in the literature in patients with stable asthma or COPD (90% to 100%) [29-31]. When SI is performed on clinical indications, for example for pneumocystis carinii diagnosis in HIV patients, success rates range from 57%-79%, which resembles our overall outcome [32].

There occurred no sudden fall in FEV_1 after SI in our LTX-patient group, including the studied patients with a low FEV_1. Beech et al also showed that no patients suffered from a severe fall in FEV_1. Even five patients with an FEV_1 (% pred) between 25%-38% were exposed to SI without side-effects or safety problems [28]. Recently it has been reported that about 50% of the patients after LTX respond with a drop in FEV_1 to a methacholine challenge, which was suggested to be predictive of early BO development [33]. This observed fall in FEV_1 after bronchial hyperresponiveness testing was not induced by SI in any patient in both the study by Beech et al as well as our study [28]. This possibly indicates a potential safe window for SI after LTX.

The numbers of total cells and neutrophil percentages were markedly elevated in SI when compared to BAL, and the percentage of neutrophils correlated fairly well in these two sampled compartments (figure 1). But as described in many other publications on the comparison of SI and BAL, also in our study a constantly proportional error was present between SI and BAL, indicating that the two methods are not interchangeable (figure 2) [24]. This apparent discrepancy between cell differentials in SI and BAL may be due to the different sites of the airways examined by the two methods [17,18,30,31]. A study in stable LTX patients comparing endobronchial biopsies, transbronchial biopsies, and BAL also suggested the existence of different, compartmentalised population of cells [21]. The higher total cell count in SI, when compared to BAL, has been found in many other pulmonary conditions, like sarcoidosis, asthma, COPD and, as shown by our results, even in healthy subjects [23,34,35].

The high percentage neutrophils obtained by SI is an important observation, because the neutrophilic granulocyte and its mediators may play a key role in the development of BO after LTX. Recent studies have shown the presence of a marked neutrophilia and high IL-8 levels in BAL when BO is present [13,18,36,37]. Furthermore, a persistently increased percentage of neutrophils is an independent predictor of mortality following LTX [15]. A recent report on induced sputum in LTX showed a good correlation between the presence of neutrophils and FEV_1 % predicted [28]. Therefore SI seems a useful tool for studying the mechanisms involved in the recruitment and subsequent activation of neutrophils in LTX recipients and their role in the development of BO.

In conclusion, SI was feasible in the majority of the LTX patients tested. SI was well tolerated and no major side-effects were noted. When performing SI in clinically stable patients after the 5th week post-LTX the success rate seems to be comparable to other indications for SI. Both techniques seem to reflect different sites of the airways, where the total number of cells, the percentage of neutrophils and IL-8 levels are markedly elevated in induced sputum of the LTX recipients compared to BALF, and compared to induced sputum of healthy subjects. These characteristics make SI

very well suited and an easy tool for further studies into the pathogenesis of BO after LTX.

REFERENCES

1. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, and Novick RJ. The registry of the international society for heart and lung transplantation: seventeenth official report. J Heart Lung Transplant 19:909-931.

2. vd Berg JW, Geertsma A, vd Bij W, Koëter GH, de Boer WJ, Postma DS, ten Vergert EM. Bronchiolitis obliterans syndrome after lung transplantation and health-related quality of life. Am J Respir Crit Care Med. 2000;161(6):1937-41.

3. Revell MP, Lewis ME, Llewellyn-Jones CG, Wilson IC, Bonser RS. Conservation of small-airway function by tacrolimus/cyclosporine conversion in the management of bronchiolitis obliterans following lung transplantation. J Heart Lung Transplant 2000;19:1219-23

4. Kelly K,.Hertz MI. 1997. Obliterative bronchiolitis. Clin Chest Med. 1997;18:319-38.

5. Scholma J, Slebos DJ, Boezen HM, Berg vd JWK, Bij vd W, Boer de W, Koëter GH, Timens W, Kauffman HF, and Postma DS. Eosinophilic granulocytes and interleukin-6 level in bronchoalveolar lavage fluid are associated with the development of obliterative bronchiolitis after lung transplantation. Am J Respir Crit Care Med 2000;162:2221-2225.

6. Cooper JD, Billingham M, Egan T, Hertz MI, Higenbottam T, Lynch J, Mauer J, Paradis I, Patterson GA, Smith C, et al. A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation. J Heart Lung Transplant. 1993;12:713-6.

7. Yousem SA, Paradis I, Griffith BP. Can transbronchial biopsy aid in the diagnosis of bronchiolitis obliterans in lung transplant recipients? Transplantation 1994;57:151-3.

8. Kramer MR, Stoehr C, Whang JL, Berry GJ, Sibley R, Marshall SE, Patterson GM, Starnes VA, Theodore J. The diagnosis of obliterative bronchiolitis after heart-lung and lung transplantation: low yield of transbronchial lung biopsy. J Heart Lung Transplant 1993;12:675-81.

9. Chaparro C, Maurer JR, Chamberlain DW, Todd TR. Role of open lung biopsy for diagnosis in lung transplant recipients: ten-year experience. Ann Thorac Surg 1995;59(4):928-32

10. Chan CC, Abi SW, Arroliga AC, Stillwell PC, Kirby TJ, Gordon SM, Petras RE, Mehta AC. Diagnostic yield and therapeutic impact of flexible bronchoscopy in lung transplant recipients. J Heart Lung Transplant 1996;15:196-205.

11. Tiroke AH, Bewig B, Haverich A. Bronchoalveolar lavage in lung transplantation. State of the art. Clin Transplant 1999;13:131-157.

12. Ward C, Whitford H, Snell G, Bao H, Zheng L, Reid D, Williams TJ, Walters EH. Bronchoalveolar lavage macrophage and lymphocyte phenotypes in lung transplant recipients. J Heart Lung Transplant, 2001;20(10):1064-1074

13. Zheng L, Walters EH, Ward C, Wang N, Orsida B, Whitford H, Williams TJ, Kotsimbos T, Snell GI. Airway neutrophilia in stable and bronchiolitis obliterans syndrome patients following lung transplantation. Thorax 2000;55:53-59.

14. Slebos DJ, Scholma J, Boezen HM, Koëter GH, van der Bij W, Postma DS, Kauffman HF. Longitudinal profile of bronchoalveolar lavage cell characteristics in patients with a good outcome after lung transplantation. Am J Respir Crit Care Med 2002;165:501-7.

15. Henke JA, Golden JA, Yelin EH, Keith FA, Blanc PD. Persistent increases of BAL neutrophils as a predictor of mortality following lung transplant. Chest 1999;115:403-9.

16. Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am J Respir Crit Care Med. 1996;153:530-4.

17. Pizzichini E, Pizzichini MM, Kidney JC, Efthimiadis A, Hussack P, Popov T, Cox G, Dolovich J, O'Byrne P, Hargreave FE. Induced sputum, bronchoalveolar lavage and blood from mild asthmatics: inflammatory cells, lymphocyte subsets and soluble markers compared. Eur Respir J 1998;11:828-34.

18. Alexis NE, Hu SC, Zeman K, Alter T, Bennett WD. Induced sputum derives from the central airways: confirmation using a radiolabeled aerosol bolus delivery technique. Am J Respir Crit Care Med 2001;164:1964-70.

19. Yousem SA, Paradis IL, Dauber JA, Zeevi A, Rabinowich H, Duquesnoy R, Hardesty R, Griffith BP. Large airway inflammation in heart-lung transplant recipients--its significance and prognostic implications. Transplantation 1990;49:654-6.

20. Yousem SA, Duncan SR, Ohori NP, Sonmez-Alpan E. Architectural remodeling of lung allografts in acute and chronic rejection. Arch Pathol Lab Med 1992;116:1175-80.

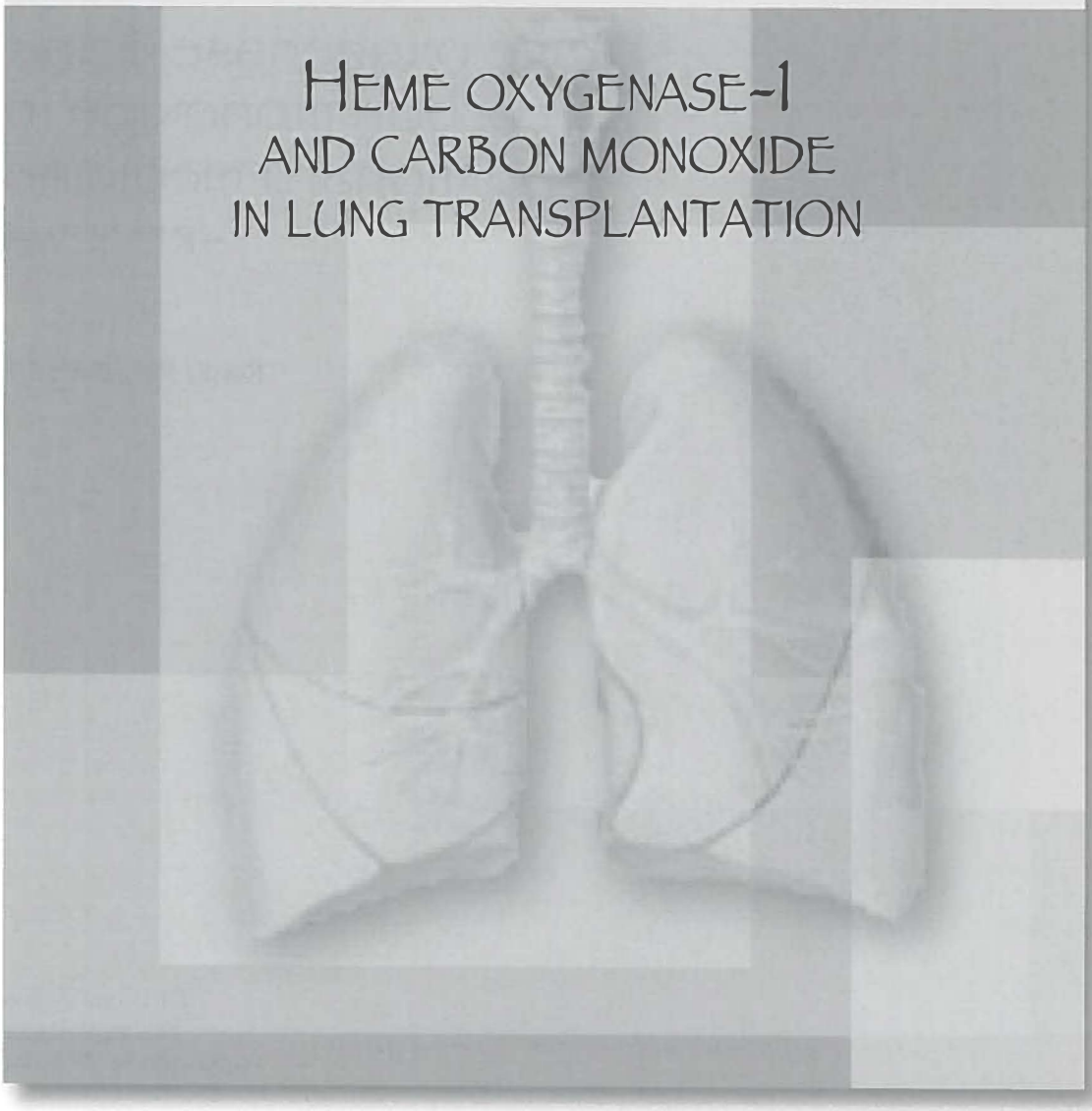
21. Ward C, Snell GI, Orsida B, Zheng L, Williams TJ, Walters EH. Airway versus transbronchial biopsy and BAL in lung transplant recipients: different but complementary. Eur Respir J 1997;10:2876-80.

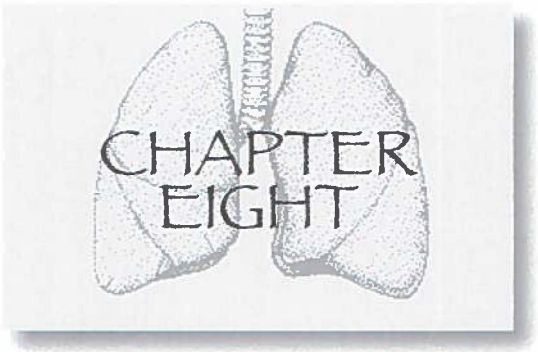
22. Zheng L, Ward C, Snell GI, Orsida B, Whitford

H, Williams TJ, Walters EH. Scar collagen deposition in the airways of allografts of lung transplant recipients. Am J Respir Crit Care Med 1997;155: 2072-7.
23. Rutgers SR, Timens W, Kaufman HF, van der Mark ThW, Koëter GH, Postma DS. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. Eur Respir J 2000;15:109-15.
24. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1(8476):307-10.
25. Trulock EP. Lung transplantation. Am J Respir Crit Care Med 1997;155:789-818.
26. Nightingale JA, Rogers DF, Barnes PJ. Effect of repeated sputum induction on cell counts in normal volunteers. Thorax 1998;53:87-90
27. Holz O, Richter K, Jörres RA, Speckin, Mucke M, Magnussen H. Changes in sputum composition between two inductions performed on consecutive days. Thorax 1998;53:83-86
28. Beech KM, Kornmann O, Lill J, Buhl R. Induced sputum cell profiles in lung transplant recipients with or without chronic rejection: correlation with lung function.Thorax 2001;56(7):557-60.
29. Hunter CJ, Ward R, Woltmann G, Wardlaw AJ, Pavord ID. The safety and success rate of sputum induction using a low output ultrasonic nebuliser. Respir Med 1999;93(5):345-8.
30. Maestrelli P, Saetta M, Di Stefano A, Calcagni PG, Turato G, Ruggieri MP, Roggeri A, Mapp CE, Fabbri LM. Comparison of leukocyte counts in sputum, bronchial biopsies, and bronchoalveolar lavage. Am J Respir Crit Care Med 1995;152:1926-31.
31. Keatings VM, Evans DJ, O'Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. Thorax 1997;52(4): 372-4.
32. Bustamante EA, Levy H. Sputum induction compared with bronchoalveolar lavage by Ballard catheter to diagnose Pneumocystis carinii pneumonia. Chest 1994;105(3):816-22.
33. Van Muylem A, Paiva M, Estenne M. Involvement of peripheral airways during methacholine-induced bronchoconstriction after lung transplantation. Am J Respir Crit Care Med 2001;164:1200-3
34. Fahy JV, Wong H, Liu J, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. Am J Respir Crit Care Med 1995;152(1):53-8.
35. Dippolito R, Foresi A, Chetta A, Castagnaro A, Malorgio R, Marangio E, Olivieri D. Eosinophils in induced sputum from asymptomatic smokers with normal lung function. Respir Med 2001;95(12): 969-74.
36. DiGiovine B, Lynch JP, Martinez FJ, Flint A, Whyte RI, Iannettoni MD, Arenberg DA, Burdick MD, Glass MC, Wilke CA, Morris SB, Kunkel SL, Strieter RM. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. J Immunol 1996;157: 4194-202.
37. Riise GC, Williams A, Kjellstrom C, Schersten H, Andersson BA, Kelly FJ. Bronchiolitis obliterans syndrome in lung transplant recipients is associated with increased neutrophil activity and decreased antioxidant status in the lung. Eur Respir J 1998;12: 82-8.

Part II.

Heme oxygenase-1 and carbon monoxide in lung transplantation





Heme oxygenase-1 and carbon monoxide in pulmonary medicine

~REVIEW~

Respir Res, 2003; 4:7

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Abstract

Heme oxygenase-1 (HO-1), an inducible stress protein, confers cytoprotection against oxidative stress *in vitro* and *in vivo*.
In addition to its physiological role in heme degradation, HO-1 may influence a number of cellular processes, including growth, inflammation, and apoptosis. By virtue of anti-inflammatory effects, HO-1 limits tissue damage in response to pro-inflammatory stimuli, and prevents allograft rejection after transplantation. The transcriptional upregulation of HO-1 responds to many agents such as hypoxia, bacterial lipopolysaccharide, and reactive oxygen/nitrogen species. HO-1, and its constitutively expressed isozyme heme oxygenase-2, catalyze the rate-limiting step in the conversion of heme to its metabolites, bilirubin-IXα, ferrous iron, and carbon monoxide (CO).
The mechanisms by which HO-1 provides protection likely involve its enzymatic reaction products. Remarkably, administration of CO at low concentrations can substitute for HO-1 with respect to anti-inflammatory and anti-apoptotic effects, suggesting a role for CO as a key mediator of HO-1 function. Chronic low level exogenous exposure to CO from cigarette smoking contributes to the importance of CO in pulmonary medicine.
The implications of the HO-1/CO system in pulmonary diseases will be discussed in this review, with an emphasis on inflammatory states.

ABBREVIATIONS

AP-1	*activator protein-1*
BALF	*bronchoalveolar lavage fluid*
CF	*cystic fibrosis*
cGMP	*cyclic 3':5'-guanosine monophosphate*
CO	*carbon monoxide*
COPD	*chronic obstructive pulmonary disease*
E-CO	*exhaled carbon monoxide*
GSH	*glutathione, reduced form*
Hb-CO	*carboxyhemoglobin*
HO-1	*heme oxygenase-1*
IL	*interleukin*
kb	*kilobase*
MAPK	*mitogen-activated protein kinase*
NF-κB	*nuclear factor κB*
NO	*nitric oxide*
OB	*obliterative bronchiolitis*
p38	*38 kilodalton protein*
ppm	*parts per million*
sGC	*soluble guanylate cyclase*
TNF-α	*tumor necrosis factor-α*

INTRODUCTION

The heme oxygenase-1/carbon monoxide (HO-1/CO) system has recently seen an explosion of research interest due to its newly discovered physiological effects. This metabolic pathway, first characterized by Tenhunen et al. [1,2], has only recently revealed its surprising cytoprotective properties [3,4]. Research in HO-1/CO now embraces the entire field of medicine where reactive oxygen/nitrogen species, inflammation, growth control, and apoptosis represent important pathophysiological mechanisms [3-6]. Indeed, the number of publications in recent years concerning HO-1 has increased exponentially, while the list of diseases and physiological responses associated with changes in HO-1 continues to expand [5].

Until now, relatively few studies have addressed the role of HO-1/CO in pulmonary medicine. Several investigators have focused on the diagnostic application of the HO-1/CO system, by measuring exhaled CO (E-CO) in various pathological pulmonary conditions, such as asthma or chronic obstructive pulmonary disease (COPD) [7]. In another experimental approach, investigators have examined the expression of HO-1 in lung tissue from healthy or diseased subjects [8,9]. This review will highlight the actions of HO-1/CO in the context of pulmonary diseases (Fig. 1), emphasizing potential protective effects against inflammation, allergic reactions, oxidative stress, endotoxin shock, apoptosis, and tumor/cell growth [10-17].

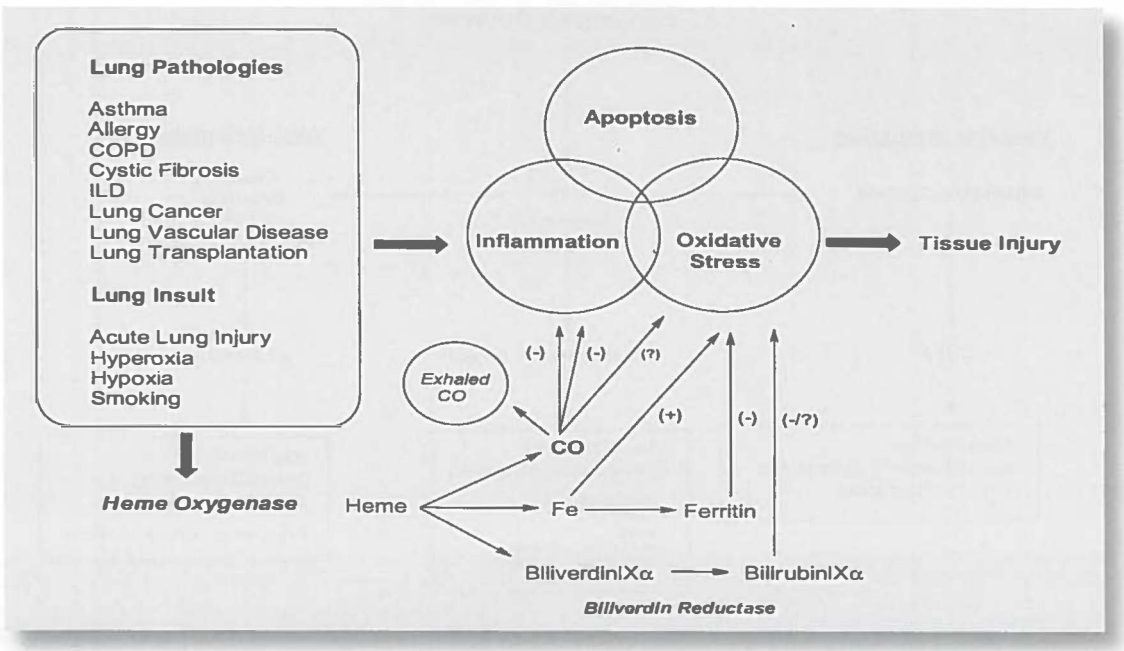
HEME OXYGENASE-1

Heme oxygenase (HO, EC 1.14.99.3) catalyzes the first and rate-limiting step in heme degradation. In the HO reaction, the oxidation of heme generates equimolar ferrous iron, biliverdin IXα, and CO. NAD(P)H: biliverdin reductase subsequently converts bilverdin IXα into bilirubin IXα [1]. The bile pigments generated during heme degra-

dation have antioxidant properties [18,19]. The liberated heme iron undergoes detoxification either by extracellular efflux or by sequestration into ferritin, an intracellular iron-storage molecule with potential cytoprotective function [20-23]. Of the three known isoforms of HO (HO-1, HO-2, and HO-3), only HO-1 responds to xenobiotic induction [24-27]. Constitutively expressed in many tissues, HO-2 occurs at high levels in nervous and vascular tissues, and may respond to regulation by glucocorticoids [25,28,29]. HO-1 and HO-2 differ in genetic origin, in primary structure, in molecular weight, and in their substrate and kinetic parameters [25,26]. HO-3 displays a high sequence homology with HO-2 but has little enzymatic activity [27]. This review will focus on the inducible, HO-1, form.

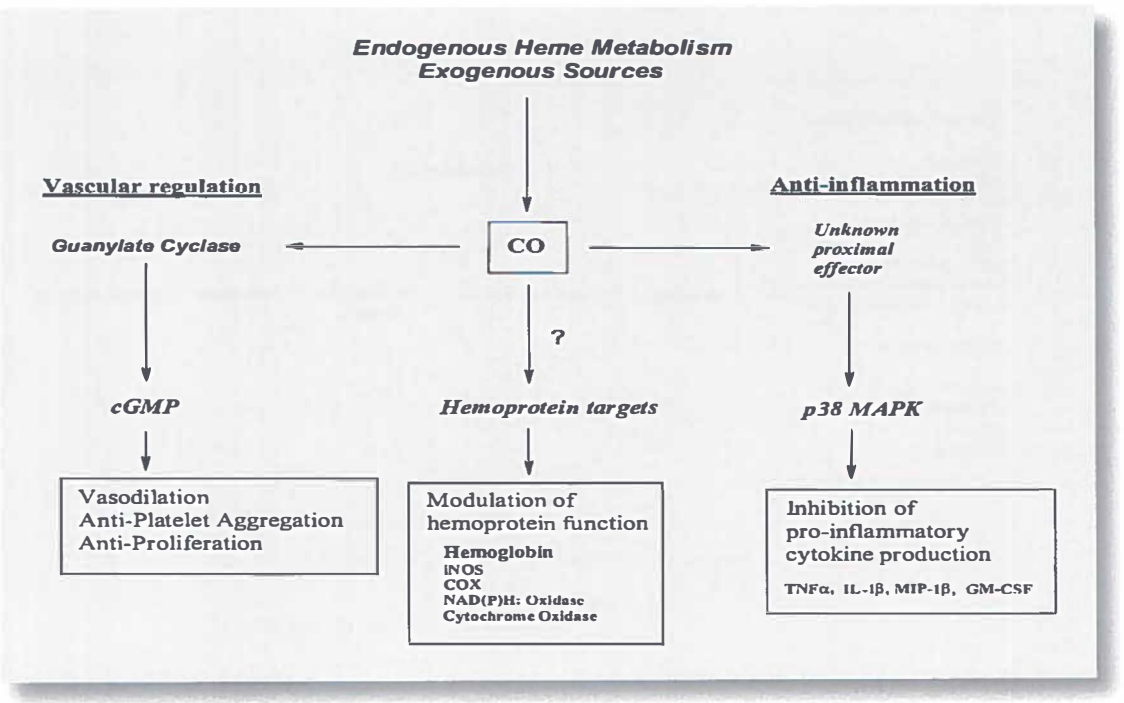
In addition to the physiological substrate heme, HO-1 responds to induction by a wide variety of stimuli associated with oxidative stress. Such inducing agents include hypoxia, hyperoxia, cytokines, nitric oxide (NO), heavy metals, ultraviolet-A (320–380 nm) radiation, heat shock, shear stress, hydrogen peroxide, and thiol (-SH)-reactive substances [3]. The multiplicity of toxic inducers suggest that HO-1 may function

FIGURE 1.
Role of HO and CO in lung diseases.
Heme oxygenase (HO) generates biliverdin-IXα, ferrous iron, and carbon monoxide (CO) from the oxidation of heme. Exhaled CO (E-CO) reflects active heme metabolism. Inflammation, oxidative stress, and apoptosis represent an axis of disease, against which both endogenous HO activity and exogenous CO exert protective effects. CO may inhibit both inflammation and apoptosis. The toxicological properties of CO imply increased pro-oxidant activity, however, the pro-/and anti-oxidant consequences of CO in the physiological range remain unclear. The bile pigments biliverdin-IXα and bilirubin-IXα have demonstrated anti-oxidant properties, though their prospective roles in modulation of inflammation and apoptosis are currently under investigation. Iron (Fe) released from HO activity returns to a transient chelatable pool, where it may potentially promote oxidative stress and apoptosis. Induction of ferritin synthesis, and sequestration of the released iron into ferritin may represent one possible detoxification pathway that limits the potential of iron in pro-apoptotic and pro-oxidative processes.



as a critical cytoprotective molecule [3,4]. Many studies have suggested that HO-1 acts as an inducible defense against oxidative stress, in models of inflammation, ischemia-reperfusion, hypoxia, and hyperoxia-mediated injury (reviewed in [3]). The mechanisms by which HO-1 can mediate cytoprotection are still poorly understood. All three products of the HO reaction potentially participate in cellular defense, of which the gaseous molecule CO has recently received the most attention [30,31]. The administration of CO at low concentrations can compensate for the protective effects of HO-1 in the presence of competitive inhibitors of HO-1 activity [32-34]. While HO-1 gene transfer confers protection against oxidative stress in a number of systems, clearly not all studies support a beneficial role for HO-1 expression. Cell-culture studies have suggested that the protective effects of HO-1 overexpression fall within a critical range, such that the excess production of HO-1 or HO-2 may be counterprotective due to a transient excess of reactive iron generated during active heme metabolism [35,36]. Thus, an important caveat of comparative studies on the therapeutic effects

FIGURE 2.
Possible mechanism(s) of CO action.
Endogenous CO arises principally as a product of heme metabolism, from the action of heme oxygenase enzymes, although a portion may arise from environmental sources such as pharmacological administration or accidental exposure, or other endogenous processes such as drug and lipid metabolism. The vasoregulatory properties of CO, including its effects on cellular proliferation, platelet aggregation, and vasodilation have been largely ascribed to the stimulation of guanylate cyclase by direct heme binding, leading to the generation of cyclic GMP. The anti-inflammatory properties of CO are associated with the downregulation of pro-inflammatory cytokine production, dependent on the selective modulation of MAPK activities. In addition to these two mechanisms, CO may potentially interact with any hemoprotein target, though the functional consequences of these interactions with respect to cellular signaling remain poorly understood.



of CO administration versus HO-1 gene delivery arises from the fact that the latter approach, in addition to producing CO, may have profound effects on intracellular iron metabolism.

HO-1 expression is primarily regulated at the transcriptional level. Genetic analyses have revealed two enhancer sequences (E1, E2) in the murine HO-1 gene located at -4 kb (E1) and -10 kbp (E2) of the transcriptional start site [37,38]. These enhancers mediate the induction of HO-1 by many agents, including heavy metals, phorbol esters, endotoxin, oxidants, and heme. E1 and E2 contain repeated stress-responsive elements, which consist of overlapping binding sites for transcription factors including activator protein-1 (AP-1), v-Maf oncoprotein, and the cap'n'collar/basic-leucine zipper family of proteins (CNC-bZIP), of which Nrf2 (NF-E2-related factor) may play a critical role in HO-1 transcription [39]. The promoter region of HO-1 also contains potential binding sites for nuclear factor κB (NF-κB), though the functional significance of these are not clear [40]. Both NF-κB and AP-1 have been identified as regulatory elements responsive to oxidative cellular stress [40,41]. In response to hyperoxic stress, AP-1 factors mediated the induction of HO-1 in cooperation with signal-transducer and activator of transcription (STAT) proteins [41]. Furthermore, a distinct hypoxia-response element (HRE), which mediates the HO-1 response to hypoxia, represents a binding site for the hypoxia-inducible factor-1 (HIF-1) [42].

CARBON MONOXIDE

The toxic properties of CO are well known in the field of pulmonary medicine. This invisible, odorless gas still claims many victims each year by accidental exposure. CO evolves from the combustion of organic materials and is present in smoke and automobile exhaust. The toxic actions of CO relate to its high affinity for hemoglobin (240-fold greater than that of O_2). CO replaces O_2 rapidly from hemoglobin, causing tissue hypoxia [43-45]. At high concentrations, other mechanisms of CO-induced toxicity may include apoptosis, lipid peroxidation, and inhibition of drug metabolism and respiratory enzyme functions [44].

Only recently has it become known that, at very low concentrations, CO participates in many physiological reactions. Where a CO exposure of 10,000 parts per million (ppm) (1% by volume CO in air) is toxic, 100–250 ppm (one hundredth to one fortieth as much) will stimulate the physiological effects without apparent toxicity [4]. The majority of endogenous CO production originates from active heme metabolism (>86%), though a portion may be produced in lipid peroxidation and drug metabolism reactions [46]. Cigarette smoking, still practiced by many lung patients, represents a major source of chronic low-level exposure to CO. Inhaled CO initially targets alveolar macrophages and respiratory epithelial cells.

The exact mechanisms by which CO acts at the molecular level remain incompletely understood. CO potentially exerts its physiological effects by influencing at least three known pathways (Fig. 2). By complexation with the heme moiety of the enzyme, CO activates soluble guanylate cyclase (sGC), stimulating the production of cyclic 3':5'-guanosine monophosphate (cGMP) [47]. The sGC/cGMP pathway mediates the effects of CO on vascular relaxation, smooth muscle cell relaxation, bronchodilation, neurotransmission, and the inhibition of platelet aggregation, coagulation, and smooth muscle proliferation [48-51]. Furthermore, CO may cause vascular relaxation by directly activating calcium-dependent potassium channels [52-54]. CO potentially influences other intracellular signal transduction pathways. The mitogen-activated protein

kinase (MAPK) pathways, which transduce oxidative stress and inflammatory signaling (i.e. response to lipopolysaccharide), may represent an important target of CO action [32,34,55,56]. An anti-apoptotic effect of CO and its relation to MAPK has recently been described. The overexpression of HO-1 or the exogenous administration of CO prevented tumor necrosis factor α (TNF-α)-induced apoptosis in murine fibroblasts [57]. In endothelial cells, the anti-apoptotic effect of CO depended on the modulation of the p38 (38 kilodalton protein) MAPK pathway [34]. The role of the remaining heme metabolites, (i.e. Fe and biliverdin IXα) in the modulation of apoptosis is currently being investigated and is beyond the scope of this review. Recent studies have reported a potent anti-inflammatory effect of CO, involving the inhibition of proinflammatory cytokine production after endotoxin stimulation, dependent on the modulation of p38 MAPK [32]. The clinical relevance of p38 MAPK lies in the possibility of modulating this pathway in various clinical conditions to downregulate the inflammatory response [58].

INVOLVEMENT OF HO-1 AND CO IN LUNG DISEASE

Oxidative stress arising from an imbalance between oxidants and antioxidants plays a central role in the pathogenesis of airway disease [59]. In lung tissue, HO-1 expression may occur in respiratory epithelial cells, fibroblasts, endothelial cells, and to a large extent in alveolar macrophages [41,60,61]. HO-1 induction in these tissues, *in vitro* and *in vivo*, responds to common causes of oxidative stress to the airways, including hyperoxia, hypoxia, endotoxemia, heavy metal exposure, bleomycin, diesel exhaust particles, and allergen exposure [4,41,61]. Induction of HO-1 or administration of CO can protect cells from these stressful stimuli [10,41]. In one of the experiments that best illustrate the protective role of CO *in vivo*, rats were exposed to hyperoxia (>98% O_2) in the absence or presence of CO at low concentration (250 ppm). The CO-treated rats showed increased survival and a diminished inflammatory response to the hyperoxia [11]. As demonstrated in a model of endotoxin-induced inflammation, the protection afforded by CO most likely resulted from the downregulated synthesis of proinflammatory cytokines (i.e. TNF-α, IL-1β) and the upregulation of the anti-inflammatory cytokine interleukin-10 (IL-10) [32]. Furthermore, increases in exhaled CO (E-CO) have been reported in a number of pathological pulmonary conditions, such as unstable asthma, COPD, and infectious lung disease; these increases may reflect increased endogenous HO-1 activity [7]. Elevated carboxyhemoglobin (Hb-CO) levels have also been reported in these same diseases in nonsmoking subjects, where both the E-CO and Hb-CO levels decrease to normal levels in response to therapy [62].

E-CO in humans originates primarily from both systemic heme metabolism, which produces CO in various tissues, and localized (lung) heme metabolism, as a result of the combined action of inducible HO-1 and constitutive HO-2 enzymatic activity. Endogenously produced or inspired CO is eliminated exclusively by respiration [63]. Elevation of E-CO may also reflect an increase in exogenous sources such as smoking or air pollution. In addition to changes in environmental factors, elevations of E-CO in lung diseases may reflect an increase in blood Hb-CO levels in response to systemic inflammation, as well as an increase in pulmonary HO-1 expression in response to local inflammation [9,62,64].

The diagnostic value of measuring E-CO remains controversial due to many conflicting reports (i.e. some reports indicate differences in E-CO measurements between disease activity and controls, and some reports do

not). The possible explanations for these discrepancies include large differences in patient populations and in the methods used for measuring E-CO, and undefined corrections for background levels of CO. Furthermore, remarkable differences arise between studies in the magnitude of the E-CO levels in the control groups as well as in treated or untreated asthma patients. When active or passive smoking occurs, or in the presence of high background levels of CO, the measurement of E-CO is not particularly useful for monitoring airway inflammation. In patients who smoke, E-CO can be used only to confirm the smoking habit [65,66]. Comparable to the beginning era of measurements of exhaled NO, a standardization in techniques and agreement on background correction should be reached for E-CO measurements, to allow proper conclusions to be drawn in this area of investigation.

ASTHMA AND ALLERGY

Asthma, a form of allergic lung disease, features an accumulation of inflammatory cells and mucus in the airways, associated with bronchoconstriction and a generalized airflow limitation. Inflammation, a key component of asthma, involves multiple cells and mediators where an imbalance in oxidants/antioxidants contributes to cell damage. Several pathways associated with oxidative stress may participate in asthma. For example, the redox-sensitive transcription factors NF-κB and AP-1 control the expression of proinflammatory mediators [59,67-69].

In light of the potential protective effects of HO-1/CO on inflammatory processes, the study of HO-1 in asthma has gained popularity. In a mouse model of asthma, HO-1 expression increased in lung tissue in response to ovalbumin aerosol challenge, indicating a role for HO-1 in asthma [70]. In a similar model of aeroallergen-induced asthma in ovalbumin-sensitized mice, exposure to a CO atmosphere resulted in a marked attenuation of eosinophil content in bronchoalveolar lavage fluid (BALF) and downregulation of the proinflammatory cytokine IL-5 [10]. This experiment showed that exogenous CO can inhibit asthmatic responses to allergens in mice.

Recent human studies have revealed higher HO-1 expression in the alveolar macrophages and higher E-CO in untreated asthmatic patients than in healthy nonsmoking controls [71,72]. Patients with exacerbations of asthma and patients who were withdrawn from inhaled steroids showed higher E-CO levels than steroid-treated asthmatics or healthy controls [73]. Higher levels of E-CO may also occur in children with persistent asthma than in healthy controls [74]. E-CO levels may correlate with functional parameters such as peak expiratory flow rate. A low rate in asthma exacerbations correlated with high E-CO, whereas normalization of the rate with oral glucocorticoid treatment resulted in a reduction of E-CO [75]. Furthermore, increased E-CO was associated with greater expression of HO-1 in airway alveolar macrophages obtained by induced sputum in untreated asthmatic patients than in controls. These asthma patients also showed higher bilirubin levels in the induced sputum, indicating higher HO activity [71]. Furthermore, patients with asthma show an increased Hb-CO level at the time of exacerbation, with values decreasing to control levels after oral glucocorticoid treatment [62]. In human asthmatics, E-CO and airway eosinophil counts decreased in response to a one-month treatment with inhaled corticosteroids [73]. In direct contrast to such studies promoting E-CO as a useful noninvasive tool for monitoring airway inflammation, other studies reported no difference in E-CO levels of asthma patients versus healthy controls, or between patients with stable and unstable asthma. In one such report, no further change in E-CO occurred in

asthma patients after a one-month treatment of inhaled corticosteroids, despite observed decreases in airway eosinophil content and bronchial responsiveness to metacholine [76]. A recent study accentuates this finding in asthma excerbations, where no decrease in E-CO of children with asthma could be detected after oral prednisolone treatment [77]. In human allergic responses, results on elevation of E-CO are also inconclusive. A clear elevation of E-CO after allergen exposure occurred in patients with asthma during the late response, and during the early response immediately after the inhalation [78]. However, another report showed that no elevation of E-CO occurred in allergen-induced asthma within 48 hours after allergen challenge [79]. Finally, increases in E-CO were measured in allergic rhinitis, correlating with seasonal changes in exposure to allergen (pollen) [80].

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Airway inflammation plays an important role in the development of COPD, characterized by the presence of macrophages, neutrophils, and inflammatory mediators such as proteinases, oxidants, and cytokines. Furthermore, the inflammatory consequences of chronic microbiological infections may contribute to the progression of the disease. The current paradigm for the pathogenesis of COPD involves imbalances in protease/antiprotease activities and antioxidant/pro-oxidant status. Proteases with tissue-degrading capacity, (i.e. elastases and matrix metalloproteinases), when insufficiently inhibited by antiproteases, can induce tissue damage leading to emphysema. Oxidants that supersede cellular antioxidant defenses can furthermore inactivate antiproteases, cause direct injury to lung tissue, and interfere with the repair of the extracellular matrix. Smoking plays an important role in both hypotheses. Cigarette smoke will act primarily on alveolar macrophages and epithelial cells, which react to this oxidative stress by producing proinflammatory cytokines and chemokines and releasing growth factors. Nevertheless, smoking cannot be the only factor in the development of COPD, since only 15–20% of smokers develop the disease [81,82].

Exposure to reactive oxygen species (from cigarette smoke or chronic infections) and an imbalance in oxidant/antioxidant status are the main risk factors for the development of COPD. To defend against oxidative stress, cells and tissues contain endogenous antioxidant defense systems, which include millimolar concentrations of the tripeptide glutathione (GSH). A close relation exists between GSH concentration and HO-1, whereby depletion of GSH augments the transcriptional regulation of HO-1 by oxidants, suggesting that the HO-1/CO system acts as a secondary defense against oxidative stress [83-86]. Accumulating clinical evidence suggests that HO-1/CO may also play an important part in COPD. Alveolar macrophages, which produce a strong HO-1 response to stimuli, may represent the main source of CO production in the airways [60,64]. Patients with COPD have displayed higher E-CO than healthy nonsmoking controls [87]. Furthermore, much higher levels of HO-1 have been observed in the airways of smokers than in nonsmokers [64]. Among subjects who formerly smoked, patients with COPD have lower HO-1 expression in alveolar macrophages than healthy subjects [88]. A microsatellite polymorphism that is linked with the development of COPD may occur in the promoter region of HO-1, resulting in a lower production of HO-1 in people who have the polymorphism. Thus, a genetically dependent downregulation of HO-1 expression may arise in subpopulations, possibly linked to increased susceptibility to oxidative stress [89-91]. Future studies on both genetic predisposition and possible therapeutic

modalities will reveal the involvement of the HO-1/CO system in COPD.

CYSTIC FIBROSIS

Cystic fibrosis (CF) involves a deposition of hyperviscous mucus in the airways associated with pulmonary dysfunction and pancreatic insufficiency, which may be accompanied by chronic microbiological infections. E-CO readings were higher in untreated versus oral-steroid-treated CF patients [92]. Furthermore, E-CO increased in patients during exacerbations of CF, correlating to deterioration of the forced expiratory volume in one second (FEV_1), with normalization of the E-CO levels after treatment [93]. E-CO levels may correlate with exhaled ethane, a product of lipid peroxidation that serves as an indirect marker of oxidative stress. Both E-CO and exhaled ethane were higher in steroid-treated and untreated CF patients than in healthy controls [94]. E-CO was higher in children with CF than in control patients. In addition to the inflammatory and oxidative stress responses to continuous infectious pressure in these patients, E-CO may possibly respond to hypoxia. E-CO increased further in CF children following an exercise test, and correlated with the degree of oxyhemoglobin desaturation, a finding suggestive of an increased HO-1 expression in CF patients during hypoxic states induced by exercise [95].

INFECTIOUS LUNG DISEASE

In patients with pneumonia, higher Hb-CO levels can be measured at the onset of illness, with values decreasing to control levels after antibiotic treatment [62]. E-CO levels were reported to be higher in lower-respiratory-tract infections and bronchiectasis, with normalization after antibiotic treatment [96,97]. Furthermore, E-CO levels in upper-respiratory-tract infections were higher than in healthy controls [74,80]. The relationship between higher measured E-CO in these infectious states and higher Hb-CO levels cannot be concluded from these studies.

INTERSTITIAL LUNG DISEASE

The role of HO-1 in the development of interstitial lung disease remains undetermined. Comparative immunohistochemical analysis has revealed that lung tissue of control subjects, patients with sarcoidosis, usual interstitial pneumonia, and desquamative interstitial pneumonia, all showed a high expression of HO-1 in the alveolar macrophages but a weak expression in the fibrotic areas [98]. The antiproliferative properties of HO-1 suggest a possible beneficial role in limiting fibrosis; however, this hypothesis is complicated by a newly discovered relation between IL-10 and HO-1. IL-10 produced by bronchial epithelial cells promotes the growth and proliferation of lung fibroblasts [99]. HO-1 expression and CO treatment have been shown to increase the production of IL-10 in macrophages following proinflammatory stimuli [32]. Conversely, IL-10 induces HO-1 production, which is apparently required for the anti-inflammatory action of IL-10 [100].

A recent report clearly shows the suppression of bleomycin-induced pulmonary fibrosis by adenovirus-mediated HO-1 gene transfer and overexpression in C57BL/6 mice, involving the inhibition of apoptotic cell death [101]. Overall, more research is needed to elucidate the mechanisms of HO-1 in interstitial lung disease and its possible therapeutic implications.

LUNG CANCER

HO-1 action may be of great importance in solid tumors, an environment that fosters hypoxia, oxidative stress, and neovascularization. HO-1 may have both pro- and antagonistic effects on tumor growth and survival. HO-1 and CO cause growth arrest in cell-culture systems and thus may represent

a potential therapeutic modality in modulating tumor growth [16]. The overexpression of HO-1 or administration of CO in mesothelioma and adenocarcinoma mouse models resulted in improved survival (>90%) as well as reduction in tumor size (>50%) [17]. Furthermore, HO-1 expression in oral squamous cell carcinomas can be useful in identifying patients at low risk of lymph node metastasis. High expression of HO-1 was detected in groups without lymph node metastasis in this report [102]. In contrast to growth arrest, HO-1 may protect solid tumors from oxidative stress and hypoxia, possibly by promoting neovascularization. In one study, zinc protoporphyrin, a competitive inhibitor of HO-1 enzyme activity, suppressed tumor growth [103].

PULMONARY VASCULAR DISEASE

CO may represent a critical mediator of the body's adaptive response to hypoxia, a common feature in pulmonary vascular disease [104]. Since CO can modulate vascular tone by inducing cGMP and large, calcium-dependent potassium channels, HO-1 and CO probably play important roles in pulmonary vascular diseases [54]. A NO-mediated HO-1 induction occurred in the hepatopulmonary syndrome during cirrhosis, associated with enhancement of vascular relaxation [105]. In portopulmonary hypertension, elevated levels of cGMP and inducible nitric oxide synthase (iNOS) expression in the vascular endothelium, and HO-1 expression in macrophages and bronchial epithelium have been described [106]. In transgenic mice models, ho-1-/-and ho-1+/+ mice did not differ in their development of pulmonary hypertension following chronic hypoxia treatment, despite the development of right ventricular dilation and right myocardial infarction in ho-1-/- mice [107]. The preinduction of HO-1 protein with chemical inducers, however, prevented the development of pulmonary hypertension in the rat lung as a consequence of chronic hypoxia treatment [108]. Transgenic mice overexpressing HO-1 in the lung were resistant to hypoxia-induced inflammation and hypertension [109]. Further research is needed to elucidate the potential role of HO-1 and CO in primary human lung vascular diseases such as primary pulmonary hypertension.

HYPEROXIC LUNG INJURY AND ACUTE RESPIRATORY DISTRESS SYNDROME

Supplemental oxygen therapy is often used clinically in the treatment of respiratory failure. Exposure to high oxygen tension (hyperoxia) may cause acute and chronic lung injury, by inducing an extensive inflammatory response in the lung that degrades the alveolar-capillary barrier, leading to impaired gas exchange and pulmonary edema [110,111]. Hyperoxia-induced lung injury causes symptoms in rodents that resemble human acute respiratory distress syndrome [112].

Hyperoxia induced HO-1 expression in adult rats but apparently not in neonatal rats, in which the expression and activities of HO-1 and HO-2 are developmentally upregulated during the prenatal and early postnatal period [113].

Both HO-1 and HO-2 potentially influence pulmonary adaptation to high O_2 levels. In one example, the adenoviral-mediated gene transfer of HO-1 into rat lungs protected against the development of lung apoptosis and inflammation during hyperoxia [114]. *In vitro* studies showed that the overexpression of HO-1 in lung epithelial cells or rat fetal lung cells caused growth arrest and conferred resistance against hyperoxia-induced cell death [15,16]. An oxygen-tolerant variant of hamster fibroblasts that moderately overexpressed HO-1 in comparison with the parent line resisted oxygen toxicity *in vitro*. The treatment of this oxygen-tolerant

strain with HO-1 antisense oligonucleotides reduced the resistance to hyperoxia. In contrast, additional, vector-mediated, HO-1 expression did not further increase oxygen tolerance in this model [115].

In vivo studies with gene-deleted mouse strains have provided much information on the roles of HO-1 and HO-2 in oxygen tolerance. Dennery et al. demonstrated that heme oxygenase-2 knockout mice (ho-2-/-) were more sensitive to the lethal effects of hyperoxia than wild-type mice [116]. In addition to the absence of HO-2 expression, however, the mice displayed a compensatory increase in HO-1 protein expression, and higher total lung HO activity. Thus, in this model, the combination of HO-2 deletion and HO-1 overexpression resulted in a hyperoxia-sensitive phenotype. Recent studies of Dennery et al. have shown that HO-1- deleted (ho-1-/-) mice were more resistant to the lethal effects of hyperoxia than the corresponding wild type [117]. The hyperoxia resistance observed in the ho-1-/- strain could be reversed by the reintroduction of HO-1 by adenoviral-mediated gene transfer [117]. In contrast, mouse embryo fibroblasts derived from ho-1-/- mice showed increased sensitivity to the toxic effects of hemin and H_2O_2 and generated more intracellular reactive oxygen species in response to these agents [118]. Both ho-1-/- and ho-2-/- strains were anemic, yet displayed abnormal accumulations of tissue iron. Specifically, ho-1-/- accumulated nonheme iron in the kidney and liver and had decreased total iron content in the lung, while ho-2-/- mice accumulated total lung iron in the absence of a compensatory increase in ferritin levels [116,119]. The mechanism(s) by which HO-1 or HO-2 deletions result in accumulation of tissue iron remain unclear. These studies, taken together, have indicated that animals deficient in either HO-1 and HO-2 display altered sensitivity to oxidative stress conditions. Aberrations in the distribution of intra- and extra-cellular iron, may underlie in part, the differential sensitivity observed [116,117].

Otterbein et al. have shown that exogenous CO, through anti-inflammatory action, may protect the lung in a rat model of hyperoxia-induced lung injury. The presence of CO (250 ppm) prolonged the survival of rats in a hyperoxic (>95% O_2) environment, and inhibited the appearance of markers of hyperoxia-induced lung injury (i.e. hemorrhage, fibrin deposition, edema, airway protein accumulation, and BALF neutrophil influx) [11]. Furthermore, in a mouse model, CO inhibited the expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in mice induced by the hyperoxia treatment. Using gene-deleted mice, Otterbein and colleagues also observed that the protection afforded by CO in this model, similar to a lipopolysaccharide-induced model of lung injury, depended on the p38 MAPK pathway (Otterbein et al., unpublished observation, as reviewed in [3]).

In direct contrast to these studies, the group of Piantadosi and colleagues reported no significant difference in the hyperoxia tolerance of rats at CO doses between 50 and 500 ppm [120]. In their model, CO did not alter the accumulation of fluid in the airway. Furthermore, CO, when applied in combination with hyperoxia, increased the activity of myeloperoxidase, a marker of airway neutrophil influx. This study also suggested that inhalation of CO (50–500 ppm) did not alter the expression of HO-1 or other antioxidant enzymes such as Manganese superoxide dismutase (MnSOD) *in vivo* [120]. Furthermore, Piantadosi and colleagues were able to induce oxygen tolerance in rats and HO-1 expression with hemoglobin treatment, but this tolerance also occurred in the presence of HO inhibitors, thereby not supporting a role for HO activity in oxygen tolerance [121]. Although no consensus

has been reached as to the protective role of CO inhalation and/or HO-1 induction in hyperoxic lung injury, human studies will be required to show if CO will supersede NO in providing a significant therapeutic benefit in the context of severe lung diseases [122]. While antioxidant therapies have been examined, until now no human studies exist on the role of HO-1 and CO in acute respiratory distress syndrome (ARDS) and bronchopulmonary dysplasia [123].

LUNG TRANSPLANTATION

Lung transplantation is the ultimate and often last therapeutic option for several end-stage lung diseases. After lung transplantation, there remains an ongoing hazardous situation in which both acute and chronic graft failure, as well as complications of the toxic immunosuppressive regimen used (i.e. severe bacterial, fungal, and viral infections; renal failure; and Epstein-Barr-virus-related lymphomas), determine the outcome [124]. The development of chronic graft failure, obliterative bronchiolitis (OB), determines the overall outcome after lung transplantation. OB, which may develop during the first months after transplantation, is the main cause of morbidity and death following the first half-year after transplantation, despite therapeutic intervention. Once OB has developed, retransplantation remains the only therapeutic option available [124,125]. Little is known about the pathophysiological background of OB. The possible determinants of developing OB include ongoing immunological allograft response, HLADR mismatch, cytomegalovirus infection, acute rejection episodes, organ-ischemia time, and recipient age [125]. OB patients displayed elevated neutrophil counts in the BALF, and evidence of increased oxidant activity, such as increased methionine oxidation in BALF protein and decreases in the ratio of GSH to oxidized glutathione in epithelial lining fluid [126,127].

So far, only very limited research data are available on the possible role for HO-1 in allograft rejection after lung transplantation. Higher HO-1 expression has been detected in alveolar macrophages from lung tissue in lung transplant recipients with either acute or chronic graft failure than in stable recipients [128]. The protective role of HO-1 against allograft rejection has been shown in other transplantation models, in which solid organ transplantation typically benefits from HO-1 modulation. A higher expression of protective genes such as HO-1 has been observed in episodes of acute renal allograft rejection [129]. Furthermore, the induction of HO-1 alleviates graft-versus-host disease [130]. Adenoviral-HO-1 gene therapy resulted in remarkable protection against rejection in rat liver transplants [131]. The upregulation of HO-1 protected pancreatic islet cells from Fas-mediated apoptosis in a dose-dependent fashion, supporting an anti-apoptotic function of HO-1 [132,133]. HO-1 may confer protection in the early phase after transplantation by inducing Th2-dependent cytokines such as IL-4 and IL-10, while suppressing interferon-γ and IL-2 production, as demonstrated in a rat liver allograft model [134].

Beneficial effects of HO-1 modulation have also been described in xenotransplantation models, in which HO-1 gene expression appears functionally associated with xenograft survival [135]. In a mouse-to-rat heart transplant model, the effects of HO-1 upregulation could be mimicked by CO administration, suggesting that HO-derived CO suppressed the graft rejection [136]. The authors proposed that CO suppressed graft rejection by inhibition of platelet aggregation, a process that facilitates vascular thrombosis and myocardial infarction.

HO-1 may also contribute to ischemic preconditioning, a process of acquired cellular protection against ischemia/reperfusion injury, as observed in guinea pig transplanted

lungs [137]. HO-1 overexpression provided potent protection against cold ischemia/reperfusion injury in a rat model through an anti-apoptotic pathway [138,139]. The induction of HO-1 in rats undergoing liver transplantation with cobalt-protoporphyrin or adenoviral-HO-1 gene therapy resulted in protection against ischemia/reperfusion injury and improved survival after transplantation, possibly by suppression of Th1-cytokine production and decreased apoptosis after reperfusion [140,141]. Until now, no reports have addressed E-CO measurements in lung transplantation, where it is possible that differences in E-CO will be found in patients with acute and chronic allograft rejection.

CONCLUSION AND FUTURE IMPLICATIONS

The evolution of CO in exhaled breath may serve as a general marker and diagnostic indicator of inflammatory disease states of the lung, though more research will be required to verify its reliability. Increases in exhaled CO presumably reflect changes in systemic and airway heme metabolic activity from the action of HO enzymes. Evidence from numerous *in vitro* and animal studies indicates that HO-1 provides a protective function in many, if not all, diseases that involve inflammation and oxidative stress. Thus, the exploitation of HO-1 for therapeutic gain could be achieved through the modulation of HO-1 enzyme activity or its up- and downstream regulatory factors, either by gene transfer, pharmacological inducers, or direct application of CO by gas administration or chemical delivery [142-145]. The CO-releasing molecules (transition metal carbonyls) developed by Motterlini et al. [144] show promise in the pharmacological delivery of CO for therapeutic applications in vascular and immune regulation. The CO-releasing molecules have been shown to limit hypertension *in vivo* and promote vasorelaxation in isolated heart and aortic rings [144].

Ultimately, the challenge remains in applying the therapeutic potentials of HO-1 to the treatment of human diseases. *In vivo* models of transplantation have shown that HO-1 gene therapy protects against allograft rejection [129,134]. Given the toxic therapy that every transplant patient receives, especially after lung transplantation, the field of transplantation medicine may bring the first frontier for human applications of HO-1 gene therapy or exogenous CO administration. The potential use of inhalation CO as a clinical therapeutic in inflammatory lung diseases has also appeared on the horizon. In one promising study, an inhalation dose of 1500 ppm CO at the rate of 20 times per day for a week produced no cardiovascular side effects [146]. Cigarette smoking and CO inhalation at identical intervals produced comparable Hb-CO levels of approximately 5%. The question of whether or not CO can be used as an inhalation therapy will soon be replaced by questions of "how much, how long, and how often?" The fear of administering CO must be weighed against the severe toxicity of the immunosuppressive agents in current use, and the often negative outcome of solid organ transplantation.

REFERENCES

1. Tenhunen R, Marver H, Schmid R: Microsomal heme oxygenase, characterization of the enzyme. J Biol Chem 1969, 244: 6388-6394.
2. Tenhunen R, Marver HS, Schmid R: The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci U S A 1968, 61: 748-755.
3. Ryter S, Otterbein LE, Morse D, Choi AM: Heme oxygenase/ carbon monoxide signaling pathways: regulation and functional significance. Mol Cell Biochem 2002, 234/235: 249-263.
4. Otterbein LE, Choi AM: Heme oxygenase: colors of defence against cellular stress. Am J Physiol Lung Cell Mol Physiol 2000, 279:L1029-L1037.
5. Morse D, Choi AM: Heme Oxygenase-1. The "emerging molecule" has arrived. Am J Respir Cell Mol Biol 2002, 27:8-16.
6. Willis D, Moore AR, Frederick R, Willoughby DA: Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat Med 1996, 2:87-90.
7. Horvath I, MacNee W, Kelly FJ, Dekhuijzen PN, Phillips M, Doring G, Choi AM, Yamaya M, Bach FH, Willis D, Donnelly LE, Chung KF, Barnes PJ: "Haemoxygenase-1 induction and exhaled markers of oxidative stress in lung diseases", summary of the ERS Research Seminar in Budapest, Hungary, September, 1999. Eur Respir J 2001, 18: 420-430.
8. Lim S, Groneberg D, Fischer A, Oates T, Caramori G, Mattos W, Adcock I, Barnes PJ, Chung KF: Expression of heme oxygenase isoenzymes 1 and 2 in normal and asthmatic airways: effect of inhaled corticosteroids. Am J Respir Crit Care Med 2000, 162:1912-1908.
9. Donnelly LE, Barnes PJ: Expression of heme oxygenase in human airway epithelial cells. Am J Respir Cell Mol Biol 2001, 24:295-303.
10. Chapman JT, Otterbein LE, Elias JA, Choi AM: Carbon monoxide attenuates aeroallergen-induced inflammation in mice. Am J Physiol Lung Cell Mol Physiol 2001, 281: L209-L216.
11. Otterbein LE, Mantell LL, Choi AM: Carbon monoxide provides protection against hyperoxic lung injury. Am J Physiol 1999, 276: L688-L694.
12. Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S: Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 1999, 103: 129-135.
13. Inoue S, Suzuki M, Nagashima Y, Suzuki S, Hashiba T, Tsuburai T, Ikehara K, Matsuse T, Ishigatsubo Y: Transfer of heme oxygenase 1 cDNA by a replication-deficient adenovirus enhances interleukin-10 production from alveolar macrophages that attenuates lipopolysaccharide-induced acute lung injury in mice. Hum Gene Ther 2001, 12: 967-979.
14. Otterbein L, Sylvester SL, Choi AM: Hemoglobin provides protection against lethal endotoxemia in rats: the role of heme oxygenase-1. Am J Respir Cell Mol Biol 1995, 13: 595-601.
15. Otterbein LE, Lee PJ, Chin BY, Petrache I, Camhi SL, Alam J, Choi AM: Protective effects of heme oxygenase-1 in acute lung injury. Chest 1999; 116: 61S-63S.
16. Lee PJ, Alam J, Wiegand GW, Choi AM: Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia. Proc Natl Acad Sci U S A 1996, 93:10393-10398.
17. Otterbein LE, Choi AMK: Carbon monoxide at low concentrations causes growth arrest and modulates tumor growth in mice [abstract]. Am J Respir Crit Care Med 2001, 163, A476.
18. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN: Bilirubin is an antioxidant of possible physiological importance. Science 1987, 235: 1043-1046.
19. Yesilkaya A, Altinayak R, Korgun DK: The antioxidant effect of free bilirubin on cumene-hydroperoxide treated human leukocytes. Gen Pharmacol 2000, 35: 17-20.
20. Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, Snyder SH: Haem oxygenase-1 prevents cell death by regulating cellular iron. Nat Cell Biol 1999, 1: 152-157.
21. Vile GF, Tyrrell RM: Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. J Biol Chem 1993, 268: 14678-14681.
22. Vile GF, Basu-Modak S, Waltner C, Tyrrell RM: Heme oxygenase-1 mediates an adaptive response to oxidative stress in human skin fibroblasts. Proc Natl Acad Sci USA 1994, 91: 2607-2610.
23. Oberle S, Schwartz P, Abate A, Schroder H: The antioxidant defense protein ferritin is a novel and specific target for pentaerithrityl tetranitrate in endothelial cells. Biochem Biophys Res Commun 1999, 261:28-34.
24. Cruse I, Maines MD: Evidence suggesting that the two forms of heme oxygenase are products of different genes. J Biol Chem 1988, 263: 3348-3353.
25. Maines MD: Heme Oxygenase: Clinical Applications and Functions. Boca Raton: CRC Press; 1992.
26. Maines MD, Trakshel GM, Kutty RK: Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. J Biol Chem 1986, 261: 411-419.
27. McCoubrey WK, Jr., Huang TJ, Maines MD: Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-

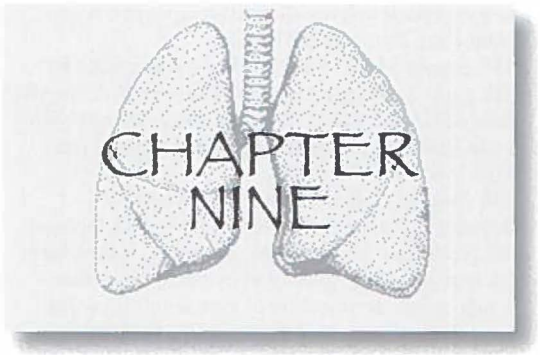
3. Eur J Biochem 1997, 247: 725-732.
28. Zakhary R, Gaine SP, Dinerman JL, Ruat M, Flavahan NA, Snyder SH: Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation. Proc Natl Acad Sci U S A 1996, 93: 795-798.
29. Raju VS, McCoubrey WK, Jr., Maines MD: Regulation of heme oxygenase-2 by glucocorticoids in neonatal rat brain: characterization of a functional glucocorticoid response element. Biochim Biophys Acta 1997, 1351: 89-104.
30. Townsend CL, Maynard R: Changing views on carbon monoxide. Clin Exp Allergy 2002, 32: 172-174.
31. Thiemermann C: Inhaled CO: deadly gas or novel therapeutic? Nat Med 2001, 7: 534-535.
32. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM: Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000, 6:422-428.
33. Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP: Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. J Immunol 2001, 166: 4185-4194.
34. Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP: Carbon monoxide generated by heme oxygenase-1 suppresses endothelial cell apoptosis. J Exp Med 2000, 192: 1015-1026.
35. Suttner DM, Sridhar K, Lee CS, Tomura T, Hansen TN, Dennery PA: Protective effects of transient HO-1 overexpression on susceptibility to oxygen toxicity in lung cells. Am J Physiol 1999, 276: L443-L451.
36. Kvam E, Hejmadi V, Ryter S, Pourzand C, Tyrrell RM: Heme oxygenase activity causes transient hypersensitivity to oxidative ultraviolet a radiation that depends on release of iron from heme. Free Radic Biol Med 2000, 28:1191-1196.
37. Alam J: Multiple elements within the 5' distal enhancer of the mouse heme oxygenase-1 gene mediate induction by heavy metals. J Biol Chem 1994, 269: 25049-25056.
38. Alam J, Camhi S, Choi AM: Identification of a second region upstream of the mouse heme oxygenase- 1 gene that functions as a basal level and inducer-dependent transcription enhancer. J Biol Chem 1995, 270: 11977-11984.
39. Choi AM, Alam J: Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 1996, 15: 9-19.
40. Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG: Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. Proc Natl Acad Sci USA 1994, 91: 5987-5991.
41. Lee PJ, Camhi SL, Chin BY, Alam J, Choi AM: AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. Am J Physiol Lung Cell Mol Physiol 2000, 279: L175-L182.
42. Lee PJ, Jiang BH, Chin BY, Iyer NV, Alam J, Semenza GL, Choi AM: Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J Biol Chem 1997, 272: 5375-5381.
43. Walker E, Hay A: Carbon monoxide poisoning. BMJ 1999, 319: 1082-1083.
44. Weaver, LK: Carbon monoxide poisoning. Crit Care Clin 1999, 15: 297-317.
45. Piantadosi CA: Carbon monoxide poisoning. N Engl J Med 2002, 347:1054-1055.
46. Vremen, HJ, Wong RJ, Stevenson DK: Carbon monoxide in breath, blood, and other tissues. In Carbon monoxide toxicity. Edited by Penney DG. Boca Raton: CRC Press, 2000; 19-60.
47. Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, Clinton Webb R, Lee ME, Nabel GJ, Nabel EG: Heme oxygenase-1 protects against vascular constriction and proliferation. Nat Med 2001, 7: 693-698.
48. Cardell LO, Ueki IF, Stjarne P, Agusti C, Takeyama K, Linden A, Nadel JA: Bronchodilatation *in vivo* by carbon monoxide, a cyclic GMP related messenger. Br J Pharmacol 1998, 124: 1065-1068.
49. Kinhult J, Uddman R, Cardell LO: The induction of carbon monoxide-mediated airway relaxation by PACAP 38 in isolated guinea pig airways. Lung 2001, 179:1-8.
50. Fujita T, Toda K, Karimova A, Yan SF, Naka Y, Yet SF, Pinsky DJ: Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. Nat Med 2001, 7: 598-604.
51. Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S: Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J Biol Chem 1997, 272: 32804-323809.
52. Koehler RC, Traystman RJ: Cerebrovascular effects of carbon monoxide. Antioxid Redox Signal 2002, 4:279-290.
53. Ingi T, Cheng J, Ronnett GV: Carbon monoxide: an endogenous modulator of the nitric oxide-cyclic GMP signaling system. Neuron 1996, 16:835-842.
54. Wang R, Wang Z, Wu L: Carbon monoxide-induced vasorelaxation and the underlying mechanisms. Br J Pharmacol 1997, 121: 927-934.
55. Kyriakis JM, Avruch J: Sounding the alarm: protein kinase cascades activated by stress and inflammation. J Biol Chem 1996, 271: 24313-24316.
56. Nick JA, Avdi NJ, Young SK, McDonald PP,

Billstrom MA, Henson PM, Johnson GL, Worthen GS: An intracellular signaling pathway linking lipopolysaccharide stimulation to cellular responses of the human neutrophil: the p38 MAP kinase cascade and its functional significance. Chest 1999, 116: 54S-55S.
57. Petrache, I, Otterbein LE, Alam J, Wiegand, GW, and Choi AM: Heme oxygenase-1 inhibits TNF-α-induced apoptosis in cultured fibroblasts. Am J Physiol Lung Cell Mol Physiol 2000, 278: L312-L319.
58. Hele DJ: Meeting report. New approaches to the modulation of inflammatory processes in airway disease models: ATS 2001, May 18-23, San Francisco. Respir Res 2001, 2: E003.
59. Rahman I, Morrison D, Donaldson K, MacNee W: Systemic oxidative stress in asthma, COPD, and smokers. Am J Respir Crit Care Med 1996, 154: 1055-1060.
60. Donnelly LE, Barnes PJ: Expression of heme oxygenase in human airway epithelial cells. Am J Respir Cell Mol Biol 2001, 24: 295-303.
61. Li N, Venkatesan MI, Miguel A, Kaplan R, Gujuluva C, Alam J, Nel A: Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. Immunol 2000, 165: 3393-3401.
62. Yasuda H, Yamaya M, Yanai M, Ohrui T, Sasaki H: Increased blood carboxyhaemoglobin concentrations in inflammatory pulmonary diseases. Thorax 2002, 57:779-783.
63. Wagner JA, Horvath SM, Dahms TE: Carbon monoxide elimination. Respir Physiol 1975, 23: 41-47.
64. Maestrelli P, El Messlemani AH, De Fina O, Nowicki Y, Saetta M, Mapp C, Fabbri LM: Increased expression of heme oxygenase (HO)-1 in alveolar spaces and HO-2 in alveolar walls of smokers. Am J Respir Crit Care Med 2001, 164: 1508-1513.
65. Middleton ET, Morice AH: Breath carbon monoxide as an indication of smoking habit. Chest 2000, 117:758-763.
66. Jarvis MJ, Russell MA, Saloojee Y: Expired air carbon monoxide: a simple breath test of tobacco smoke intake. Br Med J 1980, 281:484-485.
67. Donaldson K, Gilmour MI, MacNee W: Asthma and PM10. Respir Res 2000, 1:12-15.
68. MacNee W: Oxidative stress and lung inflammation in airways disease. Eur J Pharmacol 2001, 429: 195-207.
69. O'byrne PM, Postma DS: The many faces of airway inflammation. Asthma and chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999, 159: S41-S63.
70. Kitada O, Kodama T, Kuribayashi K, Ihaku D, Fujita M, Matsuyama T, Sugita M: Heme oxygenase-1 protein induction in a mouse model of asthma. Clin Exp Allergy 2001, 31: 1470-1477.
71. Horvath I, Donnelly LE, Kiss A, Paredi P, Kharitonov SA, Barnes PJ: Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress. Thorax 1998, 53: 668-672.
72. Harju T, Soini Y, Paakko R, Kinnula VL: Up-regulation of heme oxygenase-I in alveolar macrophages of newly diagnosed asthmatics. Respir Med 2002, 96:418-423.
73. Zayasu K, Sekizawa K, Okinaga S, Yamaya M, Ohrui T, Sasaki: Increased carbon monoxide in exhaled air of asthmatic patients. Am J Respir Crit Care Med 1997, 156:1140-1143.
74. Uasuf CG, Jatakanon A, James A, Kharitonov SA, Wilson NM, Barnes PJ: Exhaled carbon monoxide in childhood asthma. J Pediatr 1999, 135: 569-574.
75. Yamaya M, Sekizawa K, Ishizuka S, Monma M, Sasaki H, Yamara M: Exhaled carbon monoxide levels during treatment of acute asthma. Eur Respir J 1999, 13: 757-760.
76. Lim S, Groneberg D, Fischer A, Oates T, Caramori G, Mattos W, Adcock I, Barnes PJ, Chung KF: Expression of heme oxygenase isoenzymes 1 and 2 in normal and asthmatic airways: effect of inhaled corticosteroids. Am J Respir Crit Care Med 2000, 162:1912-1918.
77. Zanconato S, Scollo M, Zaramella C, Landi L, Zacchello F, Baraldi E: Exhaled carbon monoxide levels after a course of oral prednisone in children with asthma exacerbation. J Allergy Clin Immunol 2002, 109: 440-445.
78. Paredi P, Leckie MJ, Horvath I, Allegra L, Kharitonov SA, Barnes PJ: Changes in exhaled carbon monoxide and nitric oxide levels following allergen challenge in patients with asthma. Eur Respir J 1999, 13: 48-52.
79. Khatri SB, Ozkan M, McCarthy K, Laskowski D, Hammel J, Dweik RA, Erzurum SC: Alterations in exhaled gas profile during allergen-induced asthmatic response. Am J Respir Crit Care Med 2001, 164: 1844-1848.
80. Andersson JA, Uddman R, Cardell LO: Increased carbon monoxide levels in the nasal airways of subjects with a history of seasonal allergic rhinitis and in patients with upper respiratory tract infection. Clin Exp Allergy 2002, 32:224-227.
81. Rutgers SR, Timens W, Kauffman HF, Postma DS: Markers of active airway inflammation and remodelling in chronic obstructive pulmonary disease. Clin Exp Allergy 2001, 31: 193-205.
82. Croxton TL, Weinmann GG, Senior RM, Hoidal JR: Future research directions in COPD. Am J Respir Crit Care Med 2002, 165: 838-844.
83. Lautier D, Luscher P, Tyrrell RM: Endogenous

glutathione levels modulate both constitutive and UVA radiation/hydrogen peroxide inducible expression of the human heme oxygenase gene. Carcinogenesis 1992, 13: 227-232.
84. Rahman I, MacNee W: Oxidative stress and regulation of glutathione in lung inflammation. Eur Respir J 2000, 16:534-554.
85. Ewing JF, Maines MD: Glutathione depletion induces heme oxygenase-1 (HSP32) mRNA and protein in rat brain. J Neurochem 1993, 60:1512-1519.
86. Horikawa S, Yoneya, Nagashima Y, Hagiwara K, Ozasa H: Prior induction of HO-1 with glutathione depletor ameliorates the renal ischemia and reperfusion in the rat. FEBS Lett 2002,510: 221-224.
87. Montuschi P, Kharitonov SA, Barnes PJ: Exhaled carbon monoxide and nitric oxide in COPD. Chest 2001, 120: 496-501.
88. Slebos DJ, Kauffman HF, Rutgers SR, Dijkhuizen B, van Haelst PL, Choi AMK, Koeter GH, Postma DS: Haemoxygenase-1 expression in broncho-alveolar lavage fluid alveolar macrophages is diminished in patients with COPD. Eur Respir J 2002, 20 (Suppl 38): 404s.
89. Yamada N, Yamaya M, Okinaga S, Nakayama K, Sekizawa K, Shibahara S, Sasaki H: Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am J Hum Genet 2000, 66:187-195.
90. Koyama H, Geddes DM: Genes, oxidative stress, and the risk of COPD. Thorax 1998, 53: S10-S14.
91. Lomas DA, Silverman EK: The genetics of chronic obstructive pulmonary disease. Respir Res 2001, 2: 20-26.
92. Paredi P, Shah PL, Montuschi P, Sullivan P, Hodson ME, Kharitonov SA, Barnes PJ: Increased carbon monoxide in exhaled air of patients with cystic fibrosis. Thorax 1999, 54: 917-920.
93. Antuni JD, Kharitonov SA, Hughes D, Hodson ME, Barnes PJ: Increase in exhaled CO during exacerbations of cystic fibrosis. Thorax 2000, 55: 138-142.
94. Paredi P, Kharitonov SA, Leak D, Shah PL, Cramer D, Hodson ME, Barnes PJ: Exhaled ethane is elevated in cystic fibrosis and correlates with carbon monoxide levels and airway obstruction. Am J Respir Crit Care Med 2000, 16:1247-1251.
95. Horvath I, Borka P, Apor P, Kollai M: Exhaled carbon monoxide concentration increases after exercise in children with cystic fibrosis. Acta Physiol Hung 1999, 86: 237-244.
96. Biernacki WA, Kharitonov SA, Barnes PJ: Exhaled carbon monoxide in patients with lower respiratory tract infection. Respir Med 2001, 95: 1003-1005.
97. Horvath I, Loukides S, Wodehouse T, Kharitonov SA, Cole PJ, Barnes PJ: Increased levels of CO in bronchiectasis: a new marker of oxidative stress. Thorax 1998, 53: 867-870.
98. Lakari E, Pylkas P, Pietarinen-Runtti P, Paakko P, Soini Y, Kinnula VL: Expression and regulation of hemeoxygenase 1 in healthy human lung and interstitial lung disorders. Hum Pathol 2001, 32: 1257-1263.
99. Dosanjh A, Morris RE, Wan B: Bronchial epithelial cell-derived cytokine IL-10 and lung fibroblast proliferation. Transplant Proc 2001, 33: 352-354.
100. Lee TS, Chau LY: Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 2002, 8: 240-246.
101. Tsuburai T, Suzuki M, Nagashima Y, Suzuki S, Inoue S, Hasiba T, Ueda A, Ikehara K, Matsuse T, Ishigatsubo Y: Adenovirus-mediated transfer and overexpression of heme oxygen 1 cDNA in lung prevents bleomycin-induced pulmonary fibrosis via a Fas-Fas ligand-independent pathway. Hum Gene Ther 2002, 13: 1945-1960.
102. Tsuji MH, Yanagawa T, Iwasa S, Tabuchi K, Onizawa K, Bannai S, Toyooka H, Yoshida H: Heme oxygenase-1 expression in oral squamous cell carcinoma as involved in lymph node metastasis. Cancer Lett 1999, 138: 53-59.
103. Doi K, Akaike T, Fujii S, Tanaka S, Ikebe N, Beppu T, Shibahara S, Ogawa M, Maeda H: Induction of haem oxygenase-1 nitric oxide and ischaemia in experimental solid tumours and implications for tumour growth. Br J Cancer 1999, 80:1945-1954.
104. Kourembanas S: Hypoxia and carbon monoxide in the vasculature. Antioxid Redox Signal 2002, 4:291-299
105. Carter EP, Hartsfield CL, Miyazono M, Jakkula M, Morris KG Jr, McMurtry IF: Regulation of heme oxygenase-1 by nitric oxide during hepatopulmonary syndrome. Am J Physiol Lung Cell Mol Physiol 2002, 283:L346-L353.
106. Schroeder RA, Ewing CA, Sitzmann JV, Kuo PC: Pulmonary expression of iNOS and HO-1 protein is upregulated in a rat model of prehepatic portal hypertension. Dig Dis Sci 2000, 45: 2405-2410.
107. Yet SF, Perrella MA, Layne MD, Hsieh CM, Maemura K, Kobzik L, Wiesel P, Christou H, Kourembanas S, Lee ME: Hypoxia induces severe right ventricular dilatation and infarction in heme oxygenase-1 null mice. J Clin Invest 1999, 103: R23-R29.
108. Christou H, Morita T, Hsieh CM, Koike H, Arkonac B, Perrella MA, Kourembanas S: Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. Circ Res 2000, 86: 1224-1229.
109. Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, Perrella MA, Mitsialis SA, Kourembanas S: Targeted expression of heme

oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. Proc Natl Acad Sci US A 2001, 98: 8798-8803.
110. Robbins CG, Horowitz S, Merritt TA, Kheiter A, Tierney J, Narula P, Davis JM: Recombinant human superoxide dismutase reduces lung injury caused by inhaled nitric oxide and hyperoxia. Am J Physiol 1997, 272:L903-L907
111. Davis JM, Robbins CG, Anderson T, Sahgal N, Genen L, Tierney J, Horowitz S: The effects of hyperoxia, mechanical ventilation, and dexamethasone on pulmonary antioxidant enzyme activity in the newborn piglet. Pediatr Pulmonol 1995, 20: 107-111.
112. Clark JM, Lambertson CJ: Pulmonary oxygen toxicity: a review. Pharmacol Rev 1971, 23:37-133.
113. Dennery PA, Lee CS, Ford BS, Weng Y_H, Yang G, and Rodgers PA: Developmental expression of heme oxygenase in the rat lung. Pediatric Res 2003, 53: 42-47.
114. Otterbein LE, Kolls JK, Mantell LL, Cook JL, Alam J., Choi AMK: Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. J Clin Invest 1999, 103: 1047-1054.
115. Dennery PA, Sridhar KJ, Lee CS, Wong HE, Shokoohi V, Rodgers P, Spitz DR: Heme oxygenase-mediated resistance to oxygen toxicity in hamster fibroblasts. J Biol Chem 1997, 272: 14937-14943.
116. Dennery PA, Spitz DR, Yang G, Tatarov A, Lee CS, Shegog ML, Poss KD: Oxygen toxicity and iron accumulation in the lungs of mice lacking heme oxygenase-2. J Clin Invest 1998, 101: 1001-1011.
117. Dennery PA, Visner G, Weng Y-H, Nguyen X, LU F, Zander D, Yang G: Resistance to hyperoxia with heme oxygenase-1 disruption: role of iron. Free Radic Biol Med 2003, 34: 124-133.
118. Poss K.D, Tonegawa S: Reduced stress defense in heme oxygenase-1 deficient cells. 1997, Proc. Natl. Acad. Sci.USA 94: 10925-10930.
119. Poss K.D, Tonegawa S: Heme oxygenase-1 is required for mammalian iron reutilization. Proc Natl Acad Sci USA 1997, 94: 10919-10924.
120. Clayton CE, Carraway MS, Suliman HB, Thalmann ED, Thalmann KN, Schmechel DE, Piantadosi CA: Inhaled carbon monoxide and hyperoxic lung injury in rats. Am J Physiol Lung Cell Mol Physiol 2001, 281:L949-L957.
121. Taylor JL, Carraway MS, Piantadosi CA: Lung-specific induction of heme oxygenase-1 and hyperoxic lung injury. Am J Physiol 1998, 274: L582-L590.
122. Sokol J, Jacobs SE, Bohn D: Inhaled nitric oxide for acute hypoxemic respiratory failure in children and adults. Cochrane Database Syst Rev 2003, 1:CD002787.
123. Evans HE, Rosenfeld W, Jhaveri R, Concepcion L, Zabaleta I: Oxidant-mediated lung disease in newborn infants. J Free Radic Biol Med 1986, 2: 369-372.
124. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, and Novick RJ: The registry of the international society for heart and lung transplantation: seventeenth official report. J Heart Lung Transplant 2000, 19: 909-931.
125. Estenne M, Hertz MI: Bronchiolitis obliterans after human lung transplantation. Am J Respir Crit Care Med 2002, 166: 440-444.
126. Hirsch J, Elssner A, Mazur G, Maier KL, Bittmann I, Behr J, Schwaiblmair M, Reichenspurner H, Furst H, Briegel J, Vogelmeier C: Bronchiolitis obliterans syndrome after (heart-)lung transplantation. Impaired antiprotease defense and increased oxidant activity. Am J Respir Crit Care Med 1999, 160:1640-1646.
127. Behr J, Maier K, Braun B, Schwaiblmair M, Vogelmeier C: Evidence for oxidative stress in bronchiolitis obliterans syndrome after lung and heart-lung transplantation. Transplantation 2000, 69: 1856-1860.
128. Lu F, Zander DS, Visner GA: Increased expression of heme oxygenase-1 in human lung transplantation. J Heart Lung Transplant 2002, 21: 1120-1126.
129. Avihingsanon Y, Ma N, Csizmadia E, Wang C, Pavlakis M, Giraldo M, Strom TB, Soares MP, Ferran C: Expression of protective genes in human renal allografts: a regulatory response to injury associated with graft rejection. Transplantation 2002, 73: 1079-1085.
130. Woo J, Iyer S, Mori N, Buelow R: 2000. Alleviation of graft-versus-host disease after conditioning with cobalt-protoporphyrin, an inducer of HO-1. Transplantation 69: 623-633.
131. Ke B, Buelow R, Shen XD, Melinek J, Amersi F, Gao F, Ritter T, Volk HD, Busuttil RW, Kupiec-Weglinski JW: Heme oxygenase-1 gene transfer prevents CD95/Fas ligand-mediated apoptosis and improves liver allograft survival via carbon monoxide signaling pathway. Hum Gene Ther 2002, 13: 1189-1199.
132. Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH, Inverardi L: Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved *in vivo* function after transplantation. Diabetes 2001, 50:1983-1991.
133. Tobiasch E, Gunther L, Bach FH: Heme oxygenase-1 protects pancreatic beta cells from apoptosis caused by various stimuli. J Invest Med 2001, 49: 566-571.
134. Ke B, Shen XD, Melinek J, Gao F, Ritter T, Volk HD, Busuttil RW, Kupiec-Weglinski JW: Heme oxygenase-1 gene therapy: a novel immunomodula-

tory approach in liver allograft recipients? Transplant Proc 2001, 33: 581-582.
135. Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, Grey ST, Colvin RB, Choi AM, Poss KD, Bach FH: Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat Med 1998, 4:1073-1077.
136. Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP: Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. J Immunol 2001, 166:4185-4194.
137. Soncul H, Oz E, Kalaycioglu S: Role of ischemic preconditioning on ischemia-reperfusion injury of the lung. Chest 1999, 115:1672-1677.
138. Katori M, Anselmo DM, Busuttil RW, Kupiec-Weglinski JW: A novel strategy against ischemia and reperfusion injury: cytoprotection with heme oxygenase system. Transplant Immunol 2002, 9: 227-233.
139. Katori M, Buelow R, Ke B, Ma J, Coito AJ, Iyer S, Southard D, Busuttil RW, Kupiec-Weglinski JW: Heme oxygenase-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an anti-apoptotic pathway. Transplantation 2002, 73: 287-292.
140. Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, Zhao D, Zaky J, Melinek J, Lassman CR, Kolls JK, Alam J, Ritter T, Volk HD, Farmer DG, Ghobrial RM, Busuttil RW, Kupiec-Weglinski JW: Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 1999, 104:1631-1639.
141. Kato H, Amersi F, Buelow R, Melinek J, Coito AJ, Ke B, Busuttil RW, Kupiec-Weglinski JW: Heme oxygenase-1 overexpression protects rat livers from ischemia/reperfusion injury with extended cold preservation. Am J Transplant 2001, 1:121-128.
142. Immenschuh S, Ramadori G: Gene regulation of HO-1 as a therapeutic target. Biochem Pharmacol 2000, 60: 1121-1128.
143. Melo LG, Agrawal R, Zhang L, Rezvani M, Mangi AA, Ehsan A, Griese DP, Dell'Acqua G, Mann MJ, Oyama J, Yet SF, Layne MD, Perrella MA, Dzau VJ: Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygenase gene. Circulation 2002, 105:602-607.
144. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ: Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ Res 2002, 90: E17-E24.
145. Underwood DC, Osborn RR, Bochnowicz S, Webb EF, Rieman DJ, Lee JC, Romanic AM, Adams JL, Hay DW, Griswold DE: SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. Am J Physiol Lung Cell Mol Physiol 2000; 279: L895-L902.
146. Zevin S, Saunders S, Gourlay SG, Jacob P, Benowtitz NL: Cardiovascular effects of carbon monoxide and cigarette smoking. J Am Col Cardiol 2001, 38:1633-1638.



Evaluation of exhaled carbon monoxide and nitric oxide levels in lung transplant recipients

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Abstract

Background:
Bronchiolitis obliterans (BO) is the most important long-term complication after lung transplantation (LTX), and is characterised by airway inflammation both in early and advanced stages. Exhaled carbon monoxide (eCO) and nitric oxide (eNO) have both been postulated as non-invasive markers of airway inflammation, Since they may thus be helpful in the detection of BO, we evaluated their potential role as markers in BO.
Methods:
In a prospective cross-sectional design we simultaneously performed single breath eCO and eNO measurements in 97 LTX-recipients and compared these measurements to those of 14 healthy volunteers. Based upon their present bronchiolitis obliterans syndrome (BOS) stage 50 recipients were assigned to BOS 0, 23 to BOS 0-p and 24 to established BOS (stages 1-3).
Results:
LTX recipients had a significantly lower eCO level than healthy controls: median (range) 1.0 (0-3.3) ppm vs. 2.0 (0-3) ppm; $p = 0.019$. There existed no significant difference in eCO levels between absence and presence of BOS. eNO levels were similar between LTX recipients and controls: 15.5 (3.7-40.9) ppb and 13.8 (7.0-42.1) ppb respectively, but eNO levels were significantly higher in recipients with BOS when compared to BOS 0-p: 17.7 (4.0-33.4) ppb vs. 14.1 (8.1-30.6) ppb; $p= 0.03$.
Conclusions:
We were not able to show a relationship between eCO levels and the presence of BOS. eNO levels are significantly higher in patients with established BOS, but were not discriminative for the BOS 0-p stage. Longitudinal monitoring of both exhaled gases in individual patients will have to show if measuring these gases is useful in earlier detection of BO after lung transplantation than with the currently used lung function criteria.

INTRODUCTION

Lung transplantation (LTX) is an established treatment modality for patients with end-stage lung disease. The development of bronchiolitis obliterans (BO) after LTX is the commonest cause of late graft failure but may not be pathologically diagnosed until BO is in an advanced stage [1-2]. Airway inflammation in BO is characterised initially by epithelial cell injury, peribronchial inflammation, and proliferation of connective tissue causing airway occlusion. Once BO has reached its final stage, no therapeutic options are available besides re-transplantation [1].

BO is currently classified based upon the International Society of Heart and Lung Transplantation (ISHLT) criteria for the bronchiolitis obliterans syndrome (BOS). It is graded accordingly as 0, 0-p (p: probable), 1, 2 or 3 depending on the severity of the airflow limitation present relative to the baseline post-transplant FEV_1 and FEF_{25-75} (table 1) [3-5]. Although impaired lung function signifies BOS, it is likely that the development of bronchiolitis obliterans in the lung allograft takes already place before FEV_1 and FEF_{25-75} decline and thus before a measurable reduction in lung function [6].

Non-invasive evaluation of airway inflammation has become feasible by measuring exhaled carbon monoxide (eCO) and exhaled nitric oxide (eNO). In asthma, COPD, and cystic fibrosis, higher eCO levels are associated with airway inflammation [7-10]. CO, a downstream product of heme degradation, is both systemically produced by heme metabolism and by local induction, in response to airway inflammation of heme oxygenase-1 (HO-1) present in lung alveolar macrophages and epithelial cells [7,11,12]. It is yet unknown whether CO levels are increased or decreased in lung transplant recipients. In contrast to eCO, eNO has been studied previously with respect to lung allograft rejection [13-16]. eNO is probably locally produced in the airways by inducible nitric oxide synthase (iNOS) in response to neutrophilic inflammation. High eNO levels have been reported previously to reflect airway neutrophilia in stable and BOS recipients [14-15].

In this study we set out to measure eCO and eNO levels in lung allograft recipients and to compare them to values in healthy volunteers in order to investigate whether it has a potential for use in the detection of BO.

METHODS

Subjects

In a prospective cross-sectional design, LTX-recipients who visited the outpatient clinic of the University Hospital of Groningen were recruited in the period between October 2002 and January 2003. Subjects gave signed informed consent. Exclusion criteria included active or passive smoking

BOS 0	FEV_1 > 90% of baseline and FEF_{25-75} > 75% of baseline	BOS 0 *(original classification[3])*
BOS 0-p	FEV_1 81% to 90% of baseline and/or FEF_{25-75} ≤ 75% of baseline	
BOS 1	FEV_1 66% to 80% of baseline	
BOS 2	FEV_1 51% to 65 % of baseline	
BOS 3	FEV_1 < 50% of baseline	

TABLE 1.
The revised classification of bronchiolitis obliterans syndrome (BOS), according to the definition of the International Society for Heart and Lung Transplantation [4]

(assessed by questionnaire), ambient nitric oxide level > 50 ppb [17]; or an episode of acute rejection or a respiratory tract infection (both diagnosed between 3 weeks before or 3 weeks after eCO and eNO measurements). The control group consisted of 14 healthy, currently non-smoking volunteers (3F/11M) with a mean age of 51 years (range: 39-71), a mean FEV_1% predicted of 118% (101-137), and an FEV_1/VC of 79% (68-87).

Diagnostic Criteria

The LTX recipients were assigned to either BOS 0, BOS 0-p or established BOS (i.e. BOS 1-3) according to their present BOS stage, based on the ISHLT guidelines (table 1) [3,4]. FEV_1 and FEF_{25-75} were measured according to standardised guidelines [18]. Patients did not use bronchodilators within 12 hours before the pulmonary function tests.

Exhaled NO and exhaled CO measurements

eNO was measured according to published guidelines [19] with the single-breath method using a chemiluminescence analyser (CLD 700 AL; Eco Physics, Basel, Switzerland). The lower NO detection limit of the analyser was 1 ppb, with a resolution of ± 1 ppb. The sampling flow rate was 600 ml/min. Patients exhaled slowly from total lung capacity with a constant flow of 0.10 L/s against a resistance of 2 mm in diameter. Expiratory flow rate was targeted using a visual feedback display, thereby aiming at a constant flow rate of 5-6 L/minute. Three

FIGURE 1.

Boxplots (indicating: range, 25th, 50th (i.e. median: bold lines) and 75th percentile) showing single breath exhaled carbon monoxide (eCO) and nitric oxide (eNO) levels in lung transplant recipients and healthy controls. ppm = parts per million; ppb = part per billion. Numbers inside the boxplots indicate the number of patients evaluated.

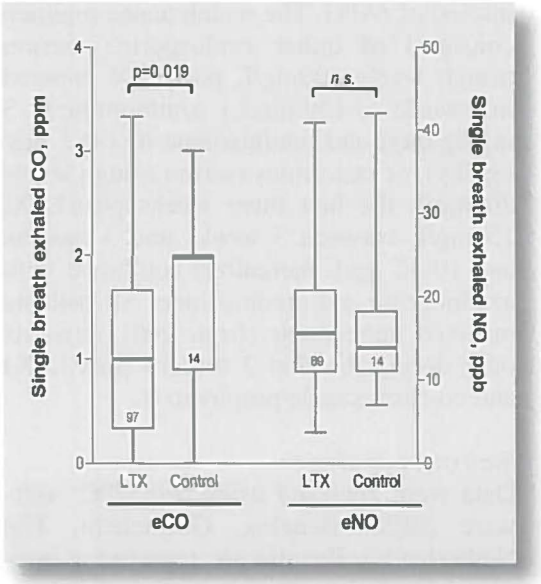
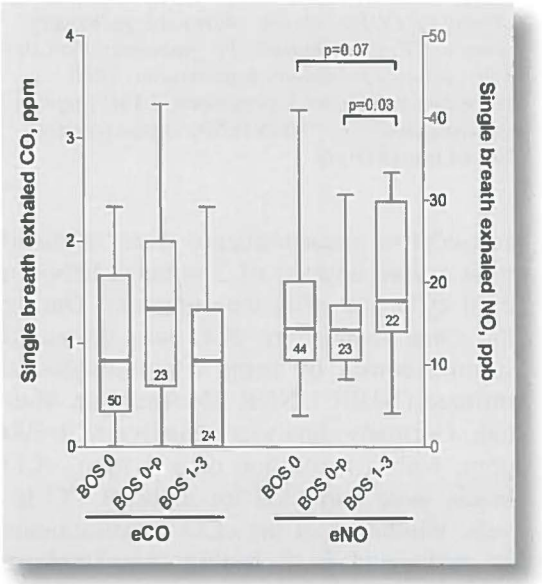


FIGURE 2.

Boxplots (indicating: range, 25th, 50th (i.e. median: bold lines) and 75th percentile) showing single breath exhaled carbon monoxide (eCO) and nitric oxide (eNO) levels in lung transplant recipients related to bronchiolitis obliterans syndrome (BOS) stage 0, 0p and 1-3. ppm = parts per million; ppb = part per billion. Numbers inside the boxplots indicate the number of patients evaluated.



Age, yrs*	47.4 (12.0)
Gender, F/M	59/38
Pre-LTX diagnosis	
COPD-AT	21
COPD	23
Bronchiectasis	6
CF	15
PF	18
PPH	5
SPH	5
LAM	3
re-lung transplantation	1
LTX-type	
Bi-lateral	57
Right-lung	20
Left-lung	16
Heart-Lung	4
Days post LTX#	1224 (23-3964)
BOS-stage [number of patients]	
0	50
0-p	23
1	13
2	4
3	7
Cyclosporine$, μg/L*	[n=29]
[number of patients]	152.7 (65.5)
Tacrolimus$, μg/L*	[n=68]
[number of patients]	12.0 (4.2)
Prednisolone dosage, mg/day*	8.9 (3.3)

TABLE 2.
*Patient characteristics. COPD-AT: α_1-antitrypsin deficiency; COPD: chronic obstructive pulmonary disease; CF: cystic fibrosis; PF: pulmonary fibrosis; PPH: primary pulmonary hypertension; SPH: secondary pulmonary hypertension; LAM: lymphangioleiomyomatosis; *mean (±SD); #mean (range); $serum trough levels.*

consecutive measurements were obtained with a time interval of 2 minutes between each eCO and eNO measurement. During the same manoeuvre eCO was measured simultaneously by using a non-dispersive infrared (NDIR UNOR 6N Analyser, Maihak, Germany) analyser sensitive to 1-500 ppm, with a resolution of ± 1 ppm. eCO levels were corrected for ambient CO levels. Validation of the eCO measurements as performed in 8 healthy non-smoking volunteers showed a good reproducibility for consecutive eCO measurements, i.e. measurements of eCO at three different time points during one day and measurements in-between days during one week (limits of agreement all $p<0.05$ as tested by one-way ANOVA). Validation of the simultaneously performed eCO and eNO measurements revealed reproducible eNO levels with or without performing eCO measurements at the same time, as well as reproducible eCO levels with or without measuring eNO at the same time (limits of agreement all $p<0.05$ as tested by one-way ANOVA). The mean of three consecutive measurements of both eCO and eNO was used for further statistical analysis. The technicians who performed the exhaled gas measurements were blinded to diagnostic endpoints.

Therapeutic protocol

Single, bi-lateral and heart-LTX were performed according to established techniques [20]. Immunosuppression after LTX included 3 doses of anti-thymocyte globulins (rATG, Merieux, dosed at 3 mg/kg) the first 10 days post-LTX. Since 2001, Basiliximab (Simulect®) 20mg on day 0 and 4 post LTX was administered as induction therapy instead of rATG. The maintenance regimen consisted of either cyclosporine (serum trough levels 400mg/L post-LTX, tapered in 3 weeks to 150 mg/L), azathioprine (1-3 mg/kg/day) and prednisolone (0.1-0.2 mg/kg/day), or tacrolimus (serum trough levels 20 mg/L the first three weeks post-LTX, 15 mg/L between 3 weeks and 3 months and 10-12 μg/L thereafter) combined with azathioprine and prednisolone. All patients received ganciclovir (from 2001 onwards only during the first 3 months post LTX) and co-trimoxazole prophylaxis.

Statistical analysis

Data were analysed using SPSS/PC+ software (SPSS Benelux, Gorinchem, The Netherlands). Results are reported as me-

dian (range) unless otherwise mentioned. Non-parametric analyses were performed with Mann-Whitney U test between groups. Correlations were performed with Spearman's rho test. Multivariate testing was performed on eCO and eNO levels, using a multivariate regression analysis with gender, age, LTX-type, days after LTX the measurements took place and the levels (cyclosporine, tacrolimus) or dosages (prednisolone) of the immunosuppressive therapy used as independent variable variables. A p-value of < 0.05 was considered statistically significant.

RESULTS

Clinical Characteristics

115 LTX-recipients approved to participate in the study. Two patients were excluded because of age <18 years, two because of active and/or passive smoking, two because of a current acute rejection episode and twelve patients were excluded because of a respiratory tract infection. Thus a total of 97 LTX-recipients could be included for further analysis, of which we had to exclude eight patients for the statistical analysis of eNO due to high ambient NO levels [17]. The characteristics of the study population are summarised in table 2.

Lung transplant recipients vs. controls

LTX-recipients had a significantly lower eCO level compared to the healthy controls: 1.0 ppm (0-3.3) vs. 2.0 ppm (0-3); $p = 0.019$ (figure 1). eNO levels were comparable between both groups: 15.5 ppb (3.7-40.9) vs. 13.8 ppb (7.0-42.1) respectively (figure 1).

BOS

eCO levels were not significantly different between stable LTX recipients (BOS 0), patients with BOS 0-p stage and established BOS (BOS stages 1-3) (figure 2). In contrast to eCO, eNO levels were significantly higher in patients with BOS 1-3 than with BOS 0-p, i.e. 17.7 ppb (4.0-33.4) and 14.1 ppb (8.1-30.6) respectively (figure 2). There existed no significant correlations between either eCO or eNO and the ratio of the present FEV_1/baseline FEV_1 (figure 3).

Factors influencing eCO and eNO levels

Monovariate analyses

The underlying disease did not contribute to the levels of both eCO and eNO. Only LTX recipients transplanted for secondary pulmonary hypertension had higher eNO levels, when compared to all other LTX indications: 17.5 ppb (14.7-40.9) vs. 15.4 (3.4-40); $p = 0.044$.

Multivariate analysis

Multivariate analysis on eCO and eNO levels showed that patients who were transplanted unilaterally had a lower eCO ($p= 0.02$). Cyclosporine serum trough levels (n=29), tacrolimus serum trough levels (n=63) and prednisolone dosages did not significantly contribute to the levels of eCO and eNO.

DISCUSSION

This study is the first one performed to assess exhaled carbon monoxide (eCO) levels after LTX. We showed that LTX-recipients had significantly lower eCO levels than healthy controls. However, we did not find a significant relationship between eCO levels and the presence of BOS. Furthermore, we confirmed previously reported data on higher eNO levels in patients with BOS than in stable LTX recipients [13,16].

We investigated the potential use of non-invasive measurements of both eCO and eNO levels in order to detect BO after LTX. We choose to study these exhaled gases in BO because they both are known to reflect inflammatory airway disease activity [7-10,14,15]. We have shown previously that high interleukin-6, interleukin-8 levels and high numbers of neutrophils and eosinophils are present in bronchoalveolar lavage fluid in stable LTX-recipients who were

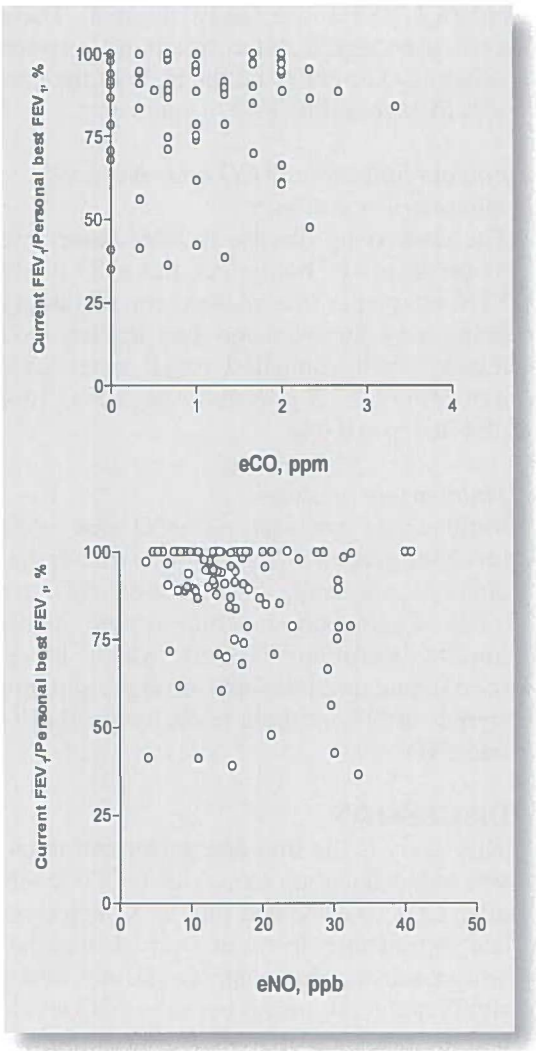


FIGURE 3.
Correlations of the ratio of the current FEV_1/ personal best post-LTX FEV_1 and single breath exhaled carbon monoxide (eCO) and nitric oxide (eNO) levels in lung transplant recipients.

shown to develop BO within one year after the bronchoalveolar lavage procedure [6]. Once established, BO is a fibrotic, inflammatory disease of the small airways characterised by neutrophilia and high IL-8 levels [21-23]. Thus we anticipated that both eCO and eNO would be increased in BOS patients. However, eCO levels were comparable between patients with and without BOS, whereas eNO levels were significantly higher in patients with BOS.

Exhaled CO

High eCO levels have been reported previously in patients with COPD and CF, diseases associated with a predominant neutrophilic airway inflammation. However, despite the association of bronchiolitis obliterans with neutrophilia, we did not find higher eCO levels in our patients having BOS [9,10]. It is possible that the airway inflammation necessary to produce sufficient amounts of CO to detect increases in our patients with BO was too low. On the other hand, we did measure minimal, but significantly elevated eNO levels in our BOS patient group. These elevated eNO levels, as reported by Gabbay et al., correlate to the airway neutrophilia present in BO, indicating that also in our BOS patients there might be indeed exist neutrophilic inflammation [16]. As previously mentioned, a slower heme metabolism due to the immunosuppressive medication used, resulting in a lower systemic CO production (and thus a lower eCO) might dilute the small amount of extra CO produced in the airways [24]. The supposedly higher CO production in the airways of patients with allograft rejection is supported by a recent study of Lu et al. who reported a higher HO-1 expression in the airways of LTX recipients with allograft rejection [25]. However, whereas a positive correlation exists between HO-1 expression and eCO levels, this correlation has until now not been demonstrated in patients after LTX [7].

We showed that the eCO levels were slightly lower in LTX recipients when compared to healthy controls. Our healthy control values (median eCO level 2 ppm) match numerous control group values reported in the literature (median values 1-3 ppm), thus these eCO levels measured seem realistic [7-10]. It can be hypothesised that the im-

munosuppressive therapy used slows down the heme metabolism, resulting in a lower systemic CO production and thus a lower eCO [25]. Furthermore it has been recently reported that eCO levels are lower in non-transplanted patients with α_1-antitrypsin deficiency when compared to both COPD and healthy controls [26]. This may contribute to the lower eCO levels, yet we were not able to show a relation between the pre-LTX diagnosis and the levels of eCO. Finally, the bronchial hyperresponsiveness present in many LTX recipients may account for the lower eCO levels as well, as is supported by a preliminary report showing reduced eCO levels in asthmatics after methacholine challenge [27-29].
Multivariate testing revealed that the immunosuppressive therapy used did not contribute to the levels of eCO and eNO. Furthermore we observed lower eCO levels in unilateral LTX recipients. This may either be an spurious finding due to multiple testing or as a result of the total amount of CO produced in the lungs, potentially contributing more extensively to the actually measured eCO levels in double LTX recipients. This clearly needs further investigation.

Exhaled NO
We were able to reproduce previous studies on higher levels of eNO present in BOS patients [13-16]. It also has been reported that the height of the exhaled NO reflects the neutrophilia present in BOS [15,16]. We therefore hypothesised that eNO levels would be elevated in BOS 0-p patients based upon the airway inflammation present [6]. However, we could not corroborate this hypothesis. Longitudinal assessments of eNO levels in individual patients will have to show if eNO measurements will help us to earlier identify the patients who will develop BO than with the current BOS criteria [4,30].
In concordance with a recent report of Verleden et al. [31] we also showed no impact of the underlying disease and of having a unilateral or double LTX on the height of the eNO levels, apart from subjects transplanted for secondary pulmonary hypertension (SPH). This patient group showed significantly higher eNO levels, when compared to all other pre-LTX diagnosis. These higher eNO levels may be due to the decreased right ventricular systolic pressure after LTX, as supported by previously observed higher eNO levels in patients with secondary pulmonary hypertension when compared to primary pulmonary hypertension patients. These patients even showed a further increase of the eNO levels after reducing the right ventricular systolic pressure by epoprostenol treatment [32].

Technical considerations
We measured both eCO and eNO simultaneously to avoid confounding due to different time-points of measuring these gases. Moreover, CO and NO are known to interact [12]. Both CO (by induction of HO-1) and NO (due to induction of iNOS) are released in response to numerous inflammatory mediators, and share a signalling action by activating soluble guanylyl cyclase. NO is furthermore capable to induce HO-1, and thereby to induce subsequent CO production. Both HO-1 and CO can furthermore inhibit iNOS activity, and thus NO production [12,33]. Based upon these interactions, theoretically, high eNO levels can result in high eCO levels, whereas lower eNO levels may accompany high eCO levels. In this study we were not able to identify such a relationship. This is not surprising, since these interactions may be too delicate to be detected by exhaled gas measurements. The order of these complicated interactions is also not known and furthermore the mode of interaction is dependent on both the kind of inflammatory mediator that induces HO-1 and iNOS as well as the cell type involved [12,33].

Whereas eNO measurements are currently standardised as described in the American Thoracic Society guidelines [19], there is yet no gold standard available as to measuring of eCO levels. In our study we used the 'single breath' method to measure eCO (a single exhalation at a constant flow rate of 5-6 L/minute after inspiration from functional residual capacity to total lung capacity (TLC) [19]). This manoeuvre may give a better reflection of the actual CO produced in the lung when compared to the 'breath-holding' manoeuvre (a fifteen-second breath-holding pause after inspiration to TLC before exhalation at a constant flow [34]), which is often used for assessing non-invasively the Hb-CO levels in smoking cessation programs [34,35]. We used an infrared-absorption technique to measure the actual eCO levels and were able to obtain reproducible eCO levels. Both electrochemical CO analysers as well as infrared-absorption techniques have been reported for the assessment of eCO levels so far [6,36]. In the near future, with increasing interest in the use of eCO measurements to assess airway inflammation, standardisation of eCO measurements is needed in order to adequately compare the outcome of different studies performed.

In conclusion, we were not able to show a relationship between eCO levels and the presence of BOS. eNO levels were significantly higher in BOS, but not discriminative for the BOS 0-p stage. Future longitudinal monitoring of both exhaled gases in individual patients will have to show if measuring these gases is useful in earlier detection of BO after lung transplantation than with the currently used lung function criteria.

ACKNOWLEDGEMENTS

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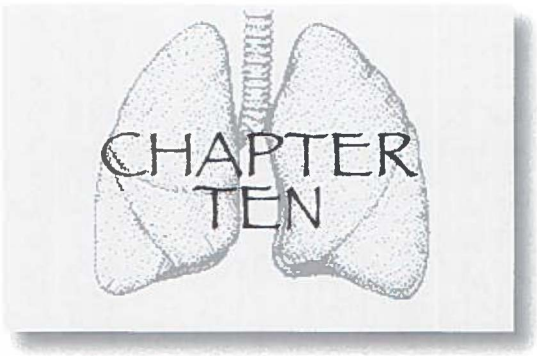
REFERENCES

1. Estenne M,.Hertz MI. Bronchiolitis obliterans after human lung transplantation. Am J Respir Crit Care Med 2002;166:440-444.
2. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, and Novick RJ. The registry of the international society for heart and lung transplantation: seventeenth official report. J Heart Lung Transplant 2000;19:909-931.
3. Cooper, J. D., M. E. Billingham, T. Egan, M. I. Hertz, T. L. Higenbottam, J. Lynch, J. Mauer, I. Paradis, G. A. Patterson, C. Smith, E. P. Trulock, C. Vreim, and S. Yousem. A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation. J Heart Lung Transplant 1993;12:713-716.
4. Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M et al. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002;21:297-310.
5. Patterson GM, Wilson S, Whang JL, Harvey J, Agacki K, Patel H et al. Physiologic definitions of obliterative bronchiolitis in heart-lung and double lung transplantation: a comparison of the forced expiratory flow between 25% and 75% of the forced vital capacity and forced expiratory volume in one second. J Heart Lung Transplant 1996;15:175-181.
6. Scholma J, Slebos DJ, Boezen HM, Berg vd JWK, Bij vd W, Boer de W, Koëter GH, Timens W, Kauffman HF, and Postma DS. Eosinophilic granulocytes and interleukin-6 level in bronchoalveolar lavage fluid are associated with the development of obliterative bronchiolitis after lung transplantation. Am J Respir Crit Care Med 2000;162:2221-2225.
7. Horvath I, Donnelly LE, Kiss A, Paredi P, Kharitonov SA, Barnes PJ. Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress. Thorax 1998;53:668-672.
8. Zayasu K, Sekizawa K, Okinaga S, Yamaya M, Ohrui T, Sasaki H. Increased carbon monoxide in exhaled air of asthmatic patients. Am J Respir Crit Care Med 1997;156:1140-1143.
9. Montuschi P, Kharitonov SA, Barnes PJ. Exhaled carbon monoxide and nitric oxide in COPD. Chest 2001;120:496-501.
10. Paredi P, Shah PL, Montuschi P, Sullivan P, Hodson ME, Kharitonov SA et al. Increased carbon monoxide in exhaled air of patients with cystic fibrosis. Thorax 1999;54:917-920
11. Maestrelli P, El Messlemani AH, De Fina O, Nowicki Y, Saetta M, Mapp C, Fabbri LM. Increased expression of heme oxygenase (HO)-1 in alveolar spaces and HO-2 in alveolar walls of smokers. Am J Respir Crit Care Med. 2001;164:1508-1513.
12. Morse D,.Choi AM. Heme oxygenase-1: the "emerging molecule" has arrived. Am.J.Respir.Cell Mol.Biol. 2002;27:8-16.
13. Fisher AJ, Gabbay E, Small T, Doig S, Dark JH, Corris PA. Cross sectional study of exhaled nitric oxide levels following lung transplantation. Thorax 1998; 53:454-458.
14. Silkoff PE, Caramori M, Tremblay L, McClean P, Chaparro C, Kesten S et al. Exhaled nitric oxide in human lung transplantation. A noninvasive marker of acute rejection. Am J Respir Crit Care Med 1998;157:1822-1828.
15. Gabbay E, Haydn WE, Orsida B, Whitford H, Ward C, Kotsimbos TC et al. In stable lung transplant recipients, exhaled nitric oxide levels positively correlate with airway neutrophilia and bronchial epithelial iNOS. Am J Respir Crit Care Med 1999;160:2093-2099.
16. Gabbay E, Walters EH, Orsida B, Whitford H, Ward C, Kotsimbos TC et al. Post-lung transplant bronchiolitis obliterans syndrome (BOS) is characterized by increased exhaled nitric oxide levels and epithelial inducible nitric oxide synthase. Am J Respir Crit Care Med 2000;162:2182-2187.
17. Corradi M, Pelizzoni A, Majori M, Cuomo A, de' ME, Pesci A. Influence of atmospheric nitric oxide concentration on the measurement of nitric oxide in exhaled air. Thorax 1998;53:673-676.
18. Quanjer P, Tammeling G, Cotes J, et al. Lung volumes and forced expiratory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur Respir J 1993;16:5-40.
19. American Thoracic Society. Standardization of Spirometry; 1994 Update. Am J Respir Crit Care Med 1995; 152:1107-1136 Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. Am J Respir Crit Care Med 1999;160:2104-2117.
20. Cooper JD. The evolution of techniques and indications for lung transplantation. Ann Surg 1990;212:249-255.
21. DiGiovine B, Lynch III JP, Martinez FJ, Flint A, Whyte RI, Iannettoni MD, Arenberg DA, Burdick MD, Glass MC, Wilke CA, Morris SB, Kunkel SL, Strieter RM. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. J Immunol 1996;157: 4194-4202.
22. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Tazelaar HD. Revision of the 1990 Wor-

king Formulation for the Classification of Pulmonary Allograft Rejection: Lung Rejection Study Group. J Heart Lung Transplant 1996;15:1-153.
23. Elssner A, Jaumann F, Dobmann S, Behr J, Schwaiblmair M, Reichenspurner H, Furst H, Briegel J, Vogelmeier C. Elevated levels of interleukin-8 and transforming growth factor-beta in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells. Munich Lung Transplant Group. Transplantation. 2000;70:362-7.
24. Krueger BA, Trakshel GM, Sluss PM, Maines MD. Cyclosporin-mediated depression of luteinizing hormone receptors and heme biosynthesis in rat testes: a possible mechanism for decrease in serum testosterone. Endocrinology. 1991;129:2647-54.
25. Lu F, Zander DS, Visner GA. Increased expression of heme oxygenase-1 in human lung transplantation. J.Heart Lung Transplant. 2002;21:1120-6.
26. Machado RF, Stoller JK, Laskowski D, Zheng S, Lupica JA, Dweik RA et al. Low levels of nitric oxide and carbon monoxide in alpha 1-antitrypsin deficiency. J.Appl.Physiol. 2002; 93:2038-43.
27. Van Muylem A, Paiva M, Estenne M. Involvement of peripheral airways during methacholine-induced bronchoconstriction after lung transplantation. Am J Respir Crit Care Med. 2001;164:1200-3.
28. Liakakos P, Snell GI, Ward C, Johns DP, Bamford TL, Williams TJ, Walters EH. Bronchial hyperresponsiveness in lung transplant recipients: lack of correlation with airway inflammation. Thorax. 1997;52:551-6.
29. White AM, Lyons NA, Ayres GA, Slivka WA, Calhoun WJ, Choi AMK, Sethi JM. Both mixed expired and end-tidal carbon monoxide are reduced during the immediate response phase of allergen and methacholine challenge in asthmatic individuals. Am J Respir Crit Care Med. 2003;167(7):A54.
30. Verleden G, Buyse B, Delcroix M, Dupont L, Van Raemdonck D, Vanhaecke J. Exhaled no (ENO) after lung transplantation (LTX): a prospective study. J Heart Lung Transplant. 2001;20:208.
31. Verleden GM, Dupont LJ, Delcroix M, Van Raemdonck D, Vanhaecke J, Lerut T, Demedts M. Exhaled nitric oxide after lung transplantation: impact of the native lung. Eur Respir J. 2003;21: 429-32.
32. Ozkan M, Dweik RA, Laskowski D, Arroliga AC, Erzurum SC. High levels of nitric oxide in individuals with pulmonary hypertension receiving epoprostenol therapy. Lung 2001;179:233-43.
33. Bouton C, Demple B. Nitric oxide-inducible expression of heme oxygenase-1 in human cells. Translation-independent stabilization of the mRNA and evidence for direct action of nitric oxide. J Biol Chem. 2000;275:32688-93.
34. Kirkham AJ, Guyatt AR, Cumming G. Alveolar carbon monoxide: a comparison of methods of measurement and a study of the effect of change in body posture. Clin Sci (Lond). 1988;74:23-8.
35. Jarvis MJ, Russell MA, Saloojee Y. Expired air carbon monoxide: a simple breath test of tobacco smoke intake. Br Med J 1980;16;281:484-5
36. Zegdi R, Perrin D, Burdin M, Boiteau R, Tenaillon A. Increased endogenous carbon monoxide production in severe sepsis. Intensive Care Med. 2002;28:793-6.



Heme oxygenase-1 expression in alveolar macrophages is elevated in patients with bronchiolitis obliterans syndrome after lung transplantation

~LETTER TO THE EDITOR~

Journal of Heart and Lung Transplantation, In press

Dirk-Jan Slebos
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Gerard H. Koëter
Wim van der Bij
Dirkje S. Postma

TO THE EDITOR,

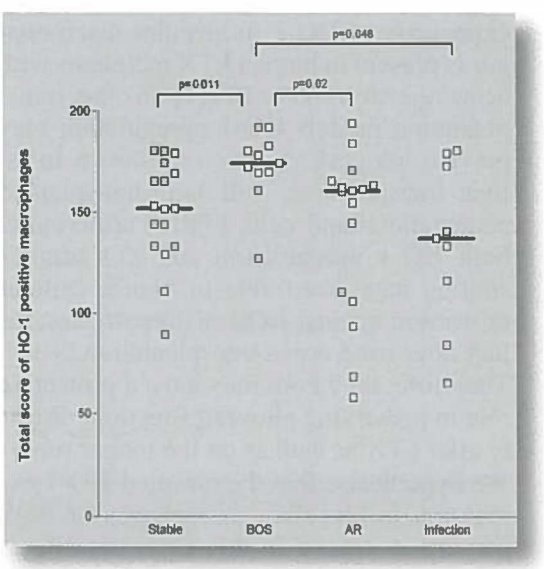
With great interest we read the recent paper of Lu et al. as recently published in this journal [1]. Lu et al. showed an increased expression of heme oxygenase-1 (HO-1) in alveolar macrophages in lung tissue samples of patients with acute and/or chronic allograft rejection after lung transplantation (LTX). Their paper speculated that the increased production of HO-1 in human lung allografts is most likely a beneficial response.

Lu et al. studied LTX patients with both acute rejection (AR) and bronchiolitis obliterans (BO) and combined them in one group. We like to extend these findings by comparing HO-1 expression in patients with AR and BO. Therefore, we retrospectively have analysed alveolar macrophage HO-1 expression in bronchoalveolar lavage fluid (BALF) samples of LTX recipients. We studied 17 stable LTX recipients, 11 recipients with bronchiolitis obliterans syndrome (BOS), 16 with a current acute rejection episode and 9 patients with a positive bacterial microbiological culture of the sampled BALF. BOS diagnosis was based on the bronchiolitis obliterans syndrome criteria formulated by the International Society of Heart and Lung Transplantation [2]. Our 11 BOS patients had a mean FEV_1 of 71.9% (± 5.8) of their own baseline value. Acute rejection was proven by histopathological examination of transbronchial biopsies taken during the same procedure [3].

The bronchoalveolar lavage (BAL) procedure and processing was performed as described previously [4]. The collected BALF was immediately placed on ice and further processed for cytospin preparation [4]. HO-1 expression was measured by immunostaining of BALF cytospins, using a HO-1 monoclonal antibody, BD Biosciences, USA. Total HO-1 expression was semi-quantitatively scored in a blinded panel by two independent observers, scores varying from negative, +, ++, to +++ as expression of increasing intensity. 400 alveolar macrophages were counted on two slides and compared to a negative control slide. Total HO-1 expression was calculated by the formula: ($HO\text{-}1^{neg}*0$)+ (HO-1+*1)+(HO-1++*2)+(HO-1+++*3). The inter-observer variability as calculated by one-way ANOVA was adequate (p=0.045). Differences in HO-1 expression were calculated using the non-parametrical Mann-Whitney-U test.

We found a significantly higher median HO-1 expression in the BOS patient group when compared to the stable LTX reci-

FIGURE 1.
*Total heme oxygenase-1 (HO-1) expression in bronchoalveolar lavage fluid from lung transplant recipients. Total HO-1 score is calculated by the formula: ($HO\text{-}1^{neg}*0$)+ (HO-1+*1)+(HO-1++*2)+(HO-1+++*3) of 2x400 scored alveolar macrophages (HO-1 expression counted as negative, or increasing positive intensity on cytospin: +, ++ and +++). Individual values are shown (□). The median value is represented by the horizontal line. BOS: Bronchiolitis obliterans syndrome; AR: Acute rejection. Stable recipients vs. both the AR and the infection group showed no significant difference.*



pients, the AR patients, and the infection patient group (figure 1). Both the AR and infection patient group showed a similar total HO-1 expression when compared to the stable LTX recipients.
Both HO-1 and its down stream product carbon monoxide (CO) have shown to possess potent cytoprotective capacities *in vitro* and *in vivo*. HO-1 can interfere in many physiological processes and has been shown to inhibit inflammatory responses, to slow down apoptosis and tumor growth, to protect against oxidative stress and to prevent allograft rejection. HO-1 is the rate-limiting enzyme in the conversion of heme to iron, bilirubin and CO. Upregulation of HO-1 (by stimuli such as hypoxia, nitric oxide, lipopolysaccharides or reactive oxygen species) results in these protective effects. The precise mechanism by which HO-1 acts is poorly understood. Remarkably most of the *in vivo* and *in vitro* effects sorted by HO-1 remain when HO-1 is inhibited and only CO is administered at very low, non-toxic, concentrations suggesting CO to be one of the active key mediators [5,6].

Until now only limited data are available on the role of HO-1 and CO in LTX allograft rejection. As shown by Lu et al., increased expression of HO-1 in alveolar macrophages is present in human LTX recipients with acute rejection and/or BO [1]. In other transplantation models HO-1 upregulation may prevent allograft rejection as shown in rat liver transplants as well as in transplanted pancreatic island cells [7,8]. Furthermore, both HO-1 upregulation and CO administration have been able to induce cellular protection against ischemia/reperfusion injury after solid organ transplantation [9,10]. Therefore, they both may have a protective role in preserving allograft function, directly after LTX as well as on the longer run.
We hypothesise that the elevated HO-1 expression in alveolar macrophages of BOS patients as shown in this letter constitutes a beneficial response to the continuous oxidative stress and inflammatory state of the airway epithelium present in BO, rather than being responsible for BO development [6,11]. It remains purely speculative why the alveolar macrophage HO-1 expression is only higher after LTX in BOS patients when compared to the AR and infection patient groups. The small difference observed may be explained by the fact that BO is a multifactorial mediated disease and is already active for months to years, with potentially many mechanisms to induce HO-1. In contrast, AR and infection episodes exist only during a few days with a simpler mode of action, and therefore fewer mechanisms to induce HO-1 when compared to BO [6,11,12]. Further prospective research seems warranted to elucidate the role and the potential therapeutic application of HO-1 and its downstream products in LTX.

REFERENCES

1. Lu F, Zander DS, Visner GA: Increased expression of heme oxygenase-1 in human lung transplantation. J Heart Lung Transplant 2002;21: 1120-1126.

2. Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M, Mallory GB, Snell GI, Yousem S. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002;21:297-310.

3. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Tazelaar HD. Revision of the 1990 Working Formulation for the Classification of Pulmonary Allograft Rejection: Lung Rejection Study Group. J Heart Lung Transplant 1996;15:1-15.

4. Slebos DJ, Scholma J, Boezen HM, Koëter GH, van der Bij W, Postma DS, Kauffman HF. Longitudinal profile of bronchoalveolar lavage cell characteristics in patients with a good outcome after lung transplantation. Am J Respir Crit Care Med 2002;165:501-507.

5. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat.Med 2000;6:422-428.

6. Morse D, Choi AM. Heme Oxygenase-1. The "emerging molecule" has arrived. Am J Respir Cell Mol Biol 2002;27:8-16.

7. Ke B, Buelow R, Shen XD, Melinek J, Amersi F, Gao F, Ritter T, Volk HD, Busuttil RW, Kupiec-Weglinski JW. Heme Oxygenase 1 Gene Transfer Prevents CD95/Fas Ligand-Mediated Apoptosis and Improves Liver Allograft Survival via Carbon Monoxide Signaling Pathway. Hum Gene Ther 2002;13:1189-99.

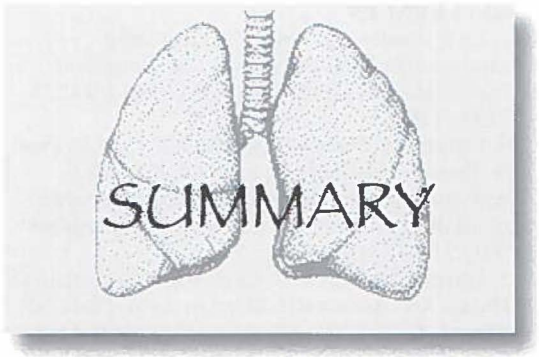
8. Tobiasch E, Gunther L, Bach FH. Heme oxygenase-1 protects pancreatic beta cells from apoptosis caused by various stimuli. J Investig Med 2001;49:566-71.

9. Katori M, Buelow R, Ke B, Ma J, Coito AJ, Iyer S, Southard D, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an antiapoptotic pathway. Transplantation 2002;73: 287-92.

10. Zhang X, Shan P, Otterbein LE, Alam J, Flavell RA, Davis RJ, Choi AM, Lee PJ. Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. J Biol Chem 2003;278:1248-58.

11. Estenne M,.Hertz MI. Bronchiolitis obliterans after human lung transplantation. Am J Respir Crit Care Med 2002;166:440-444.

12. Katori M, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 system in organ transplantation. Transplantation. 2002;74:905-12.



Bronchiolitis obliterans (BO) after Lung transplantation (LTX) remains the major long-term complication with respect to morbidity and mortality after LTX. However, there is little knowledge of the pathophysiology, it is difficult to diagnose and there is no satisfying treatment for BO in human lung transplant recipients. In **Part I** of this thesis we set out to investigate both the normal airway changes after lung transplantation as well as changes due to bronchiolitis obliterans. Therefore, at first we examined the normal airway cellular changes during an uncomplicated course after LTX by using bronchoalveolar lavage fluid analysis **(chapter 2)**. Thereafter, we evaluated the airway inflammatory characteristics in both acute and chronic allograft rejection **(chapter 3)**, and defined patients who will be at risk to develop BO in the near future based upon these airway cellular and soluble characteristics **(chapter 4)**. With the introduction of newer immunosuppressive agents, with potential better treatment results, we examined in **chapter 5** the influence of two different immunosuppressive treatment modalities on the cellular constituents of the lung. In **chapter 6** we demonstrated the clinical utility of the immunological bronchoalveolar lavage procedure after LTX, and in **chapter 7** we investigated the feasibility of sputum induction for examining the airway inflammatory characteristics in lung transplant recipients. In **Part II** of

this thesis, we investigated the potential of the heme oxygenase-1 (HO-1) and carbon monoxide (CO) system with respect to their significance in LTX. Therefore, in chapter 8 the HO-1 and CO system is reviewed with respect to pulmonary medicine in general and its potential in cytoprotection and immunosuppression. In the next two chapters we investigated the endogenous activity of the HO-1/CO system, by measuring both exhaled CO **(chapter 9)** and BALF alveolar macrophage HO-1 expression in LTX recipients **(chapter 10)**.

CHAPTER 2

In order to define what constitutes an abnormal cellular BALF-profile, information on a 'normal' BALF-profile is needed i.e. findings in patients without further pathological airway conditions after LTX. It is known that BALF-results from patients after LTX are incomparable to BALF-results from healthy subjects. In this chapter we set out to define the 'normal' cellular profile of BALF of patients after LTX without any signs or symptoms of accompanying airway pathology and evaluated its course during the first two years after LTX. From this study we conclude that a decrease in total cell count and $CD3^+$ lymphocytes, and an increase in CD4/CD8 ratio after LTX represent the natural course of cell numbers in BALF in patients without pathological airway conditions after LTX. We introduced these control values as a tool for diagnosing patients with pulmonary complications after LTX and for the follow-up of treatment regimens. Dynamic post-LTX profiles have to be taken into account when research is performed or clinical interpretations are made on BALF cellular characteristics after LTX.

CHAPTER 3

In this chapter we studied the feasibility of BALF analysis in detecting acute and chronic allograft rejection. Furthermore we tried to obtain more insight in the BALF lymphocyte population and soluble compounds during acute rejection and BO after LTX. Our study showed a possible diagnostic role for cellular and soluble analysis of BALF, since it may detect BO and acute rejection after LTX. BALF neutrophilia and an elevated IL-8 are associated with established BO. When using a cut-off point of >3% neutrophils in the lavage, the sensitivity for BO is 87.0% and the specificity 77.6%. The sensitivity of interleukin-8 (IL-8) for BO when using a cut-off point of >71.4 pg/mL is 74.5%, the specificity 83.3%. Furthermore, when BALF analysis reveals a lymphocytosis, one should beware of acute rejection. BALF lymphocyte subtypes (e.g. $CD4^+$HLADR, $CD8^+$HLADR, $CD8^+CD45^+$, $CD4^+CD25^+$) are already highly activated in stable patients just after LTX. This increased activation becomes even more pronounced in BO, but not in acute rejection. Thus, BALF analysis can thus give us better insight into the pathophysiological mechanisms underlying acute and chronic allograft rejection. However, given the imperfect sensitivity and specificity of the investigated markers, more specific BALF markers related to these pathological conditions need to be identified.

CHAPTER 4

After defining the normal BALF cellular characteristics after LTX, and after describing the changes that occur during established BO, we investigated the predictive value of bronchoalveolar lavage fluid with respect to future development of BO. In a prospective cohort study we investigated the relation between changes in leukocyte and differential counts and soluble compounds of BALF early after LTX and the development of BO later on. By means of logistic regression analysis we showed that a higher total cell count, higher neutrophilic granulocyte and lymphocyte counts, the presence of eosinophilic granulocytes, and

higher levels of IL-6 and IL-8 were significantly associated with an increased risk to develop OB. We conclude that monitoring of these indices will contribute to a better identification and management of the group of patients at risk for developing OB within a year. Future studies are needed to investigate if adjustment of the treatment regimen at this early time point in these 'patients at risk' can normalise the above mentioned aberrant BALF parameters as well as delay or even prevent the development of BO.

CHAPTER 5

Both established and novel immunosuppressive agents have a variety in routes of action to prevent allograft rejection. Therefore they might differently affect the airway inflammatory cell population after LTX. To assess these possible differences we studied the BALF cellular characteristics in two different immunosuppressive regimens used after LTX. Between two different immunosuppressive protocols a marked difference in the airway lymphocyte population was observed a few weeks after LTX. The immunosuppressive protocol containing basiliximab, tacrolimus and azathioprine showed a favourable lymphocyte subset with respect to immunocompetence when compared to a protocol containing antithymocyte globulins, cyclosporine and azathioprine. This was best shown by a higher percentage of $CD4^+$ and a lower percentage of $CD8^+$ lymphocytes (resulting in a much higher CD4/CD8 ratio) and furthermore a much lower $CD4^+CD25^+$ percentage early post-LTX for the first protocol. We also observed a beneficial trend in overall- and graft survival for the first protocol. The differences in cellular composition were not associated with more allograft complications after LTX. The differences in cellular composition may explain the better graft- and overall survival attributed to these new immunosuppressive agents.

CHAPTER 6

In this chapter we demonstrated the clinical application of the immunological BAL procedure. A LTX recipient who was transplanted because of end stage pulmonary disease due to sarcoidosis showed recurrence of the sarcoidosis in the transplanted allograft. This diagnosis was already suspected by BALF analysis before the recurrent sarcoidosis was detected and confirmed by lung biopsies.

CHAPTER 7

Frequent monitoring of patients after LTX seems necessary to obtain more insight into the slowly developing process of BO. Therefore, we studied the feasibility of sputum induction in LTX recipients. Sputum induction is thought to be less invasive when compared to the bronchoalveolar lavage procedure, and is therefore potentially more suitable for repetitive sampling. In addition we compared its cellular composition and level of IL-8 with values in BALF. Overall, sputum induction was successful in 73%. The success rate in clinically stable patients after the first month post-LTX was 93%. Side effects were absent. Total cell counts, neutrophil percentages and IL-8 levels were much higher in sputum than in BALF. Although the neutrophil percentage in BALF and induced sputum in the LTX patients were positively correlated, the methods are not interchangeable as shown by the applied Bland and Altman comparison. We conclude that sputum induction is feasible, well tolerated, and without important side effects in stable patients after the first month post LTX. Therefore, induced sputum may be a useful tool to study more frequently inflammatory changes of the airways after LTX, and because of the large quantity of neutrophils sampled, especially for further studies on BO.

CHAPTER 8

In this review, the enzyme heme oxygenase-

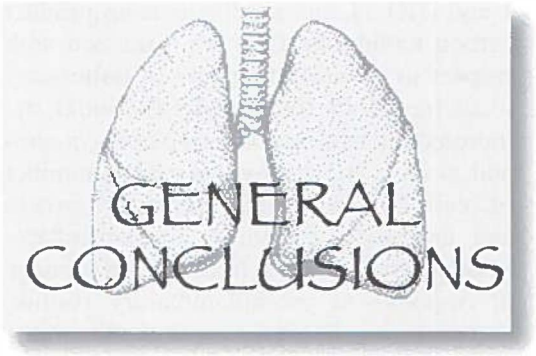
1 and (HO-1), and its downstream product carbon monoxide (CO) are discussed with respect to its protective role in pulmonary medicine. Both HO-1 and CO confer cytoprotection against oxidative stress *in vitro* and *in vivo*. HO-1 may influence a number of cellular processes, including growth and apoptosis. By virtue of anti-inflammatory effects, HO-1 limits tissue damage in response to pro-inflammatory stimuli, and prevents allograft rejection after transplantation. The mechanism by which HO-1 provides protection likely involves its enzymatic reaction products CO, biliverdin and iron. Remarkably, administration of CO at low concentrations *in vitro* and *in vivo* can substitute for HO-1 with respect to its anti-inflammatory and anti-apoptotic effects, suggesting a role for CO as a key mediator of HO-1 function. Future therapeutic administration of low-dose CO might be a new approach to inflammatory diseases and to transplantation medicine.

CHAPTER 9

To evaluate a possible pathophysiological role for CO in BO, we evaluated exhaled CO (eCO) levels and nitric oxide (eNO) levels in lung allograft recipients and healthy volunteers in a cross-sectional design. Where eCO has not yet been studied with respect to BO, eNO levels have shown in previous studies to correlate with bronchiolitis obliterans syndrome (BOS). In conclusion our study showed that eCO levels are generally lower in patients after LTX when compared to healthy volunteers. However, eCO did not differentiate potential (BOS 0-p) or established BOS from the stable recipients. eNO levels were, as expected, higher in patients with BOS, yet the eNO levels were also not discriminative for the BOS 0-p stage. eCO seems not useful in detecting BO. Further studies are needed to investigate a possible role of eCO in LTX-recipients, including both longitudinal evaluation of BO patients as well as other pathological airway conditions like acute rejection and infectious disease.

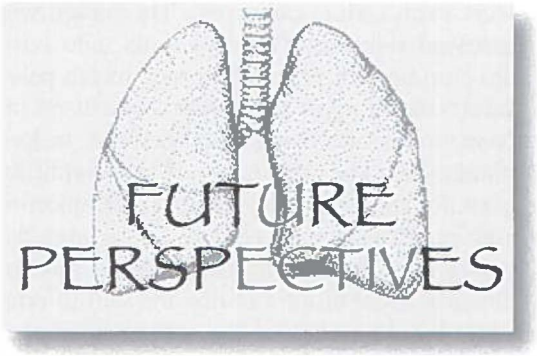
CHAPTER 10

In this chapter we performed an inventory analysis on the HO-1 expression of BALF alveolar macrophages in patients with BO after LTX, acute rejection, infection and stable recipients. By semi-quantitative examination we were able to show a significantly higher HO-1 expression for the BO patient group, when compared to stable LTX recipients. Both the acute rejection and infection patients groups showed a similar HO-1 expression when compared to the stable LTX recipients. The elevated HO-1 expression in BOS patients might be a response to the continuous oxidative stress and inflammatory state of the airway epithelium present in BO, rather than a conditioned elevated HO-1 expression as a cause of BO development. Further prospective research seems warranted to elucidate the role of HO-1 and its downstream products in LTX, most importantly with respect to its potential therapeutic applications.



Bronchiolitis obliterans (chronic allograft rejection) remains the major long-term complication with respect to morbidity and mortality after lung transplantation. However, there is little knowledge of the pathophysiology, no sensitive diagnostic tool and no satisfying treatment for bronchiolitis obliterans in human lung transplant recipients. Bronchoalveolar lavage has held out promise as a very useful tool in understanding the pathophysiology and ultimately as a diagnostic tool in lung allograft dysfunction. In this thesis we were able to assess the normal bronchoalveolar lavage fluid cellular profile after lung transplantation and introduced these values as reference values for research and clinical management purposes. We showed that the normal cellular profile changes considerably during the first two years after transplantation even in a stable state post lung transplantation. After having defined this normal time-dependent cellular lavage fluid profile, we observed that bronchiolitis obliterans is dominated by a neutrophilia and high interleukin-8 cytokine levels in the lavage fluid. When using a cut-off point of >3% neutrophils in the lavage, the sensitivity for bronchiolitis obliterans is 87.0% and the specificity 77.6%. Furthermore, we observed activated lavage lymphocyte subtypes in bronchoalveolar lavage fluid of stable lung transplant recipients, while in objectified bronchiolitis obliterans the levels of activated lymphocytes

were even further enhanced. Thereafter, we assessed risk factors for patients who will develop bronchiolitis obliterans in the near future based upon parameters measured in bronchoalveolar lavage fluid just post transplantation. The presence of eosinophilic granulocytes, high levels of interleukin-6 and interleukin-8 appeared to be significantly associated with an increased risk to develop bronchiolitis obliterans within one year. We also studied the influence of the immunosuppressive protocol used. The airway cellular characteristics appeared to be strongly different between an immunosuppressive protocol containing basiliximab, tacrolimus and azathioprine when compared to a protocol containing anti-thymocyte globulins, cyclosporine and azathioprine. There existed a beneficial trend in patient- and graft survival with the first protocol. We propose that the observed cellular differences may explain the better graft- and overall survival, which are attributed to the newer immunosuppressive agents (i.e. basiliximab and tacrolimus). The bronchoalveolar lavage procedure appears to be a useful tool in a research setting for better understanding of the inflammatory process underlying bronchiolitis obliterans. In addition, we demonstrated the clinical utility of the immunological bronchoalveolar lavage procedure by describing its use in the diagnosis of recurrent sarcoidosis in the transplanted lung. Because of the relative invasiveness of the bronchoalveolar lavage procedure and therefore the unsuitability for more frequent sampling, we also investigated the feasibility of sputum induction. This less invasive technique was well feasible in patients after lung transplantation. The success rate of sputum induction in clinically stable patients after the first month post-transplantation was 93% and without any side effects. With respect to new treatment options for patients after lung transplantation, we have discussed the pathophysiological and treatment potentials of both heme oxygenase-1 and its downstream product carbon monoxide. With respect to a potential pathophysiological role in bronchiolitis obliterans we showed a higher heme oxygenase-1 expression in bronchoalveolar lavage alveolar macrophages in patients with bronchiolitis obliterans. However, we were not able to show a relation between exhaled carbon monoxide levels in our lung transplant population and the presence of bronchiolitis obliterans. Based upon *in vitro* and *in vivo* results so far, further research with respect to the therapeutic applications of both heme oxygenase-1 modulation and carbon monoxide inhalation applied to human lung transplantation seems warranted.



Twenty years of bronchiolitis obliterans (BO) research after human lung transplantation (LTX) has provide insight in that BO is a multifactorial mediated and incurable disorder. Furthermore, has the exact pathophysiology of BO still to be unravelled. Frequent monitoring of our lung transplant recipients seems necessary to gain better insight into the process of the often slow developing BO. This monitoring will have to be performed by using our conventional diagnostic tools i.e. pulmonary function testing and routine bronchoscopies. However, we also have to extend our diagnostic strategies to reinforce our research to learn more about the pathophysiology of BO with the ultimate goal of preventing or stopping chronic transplant rejection. Some possible avenues are being described below.

MULTIPLE STRATEGY TESTING

With respect to BO diagnosis and pathophysiology we will focus the next years on multiple strategy testing by combining the current available diagnostic tools focussing on smaller airways function, the location where BO takes place. As has been shown previously, combining different diagnostic modalities can result in detection of BO in an earlier phase [1]. We will apply BOS-grading, BALF characteristics, exhaled NO, single breath N_2, bronchial hyperresponsiveness testing and expired high-resolution computed tomography and try to

develop a predictive diagnostic model for early detection of BO [2,3].

EXHALED GASES

To extend our results of chapter 9, we will measure both exhaled carbon monoxide (CO) and exhaled nitric oxide (NO) with respect to the development of BO in a longitudinal design. Since both gases have been shown to reflect airway inflammation [4,5], we will assess whether measurement of both exhaled gases contributes to a better diagnosis of BO. Furthermore we will investigate the relation of eCO levels to the local airway HO-1 activity.

$CD4^+CD25^+$

In the next years we will also try to focus on the role of the $CD4^+CD25^+$ lymphocytes in the pathogenesis of BO after LTX. A particular subset of these cells, the so-called $CD4^+CD25^{high}$ T-lymphocytes has been shown to play a critical role in the induction of graft tolerance and graft acceptance after LTX. These $CD4^+CD25^{high}$ T-cells have been shown to produce both interleukin-10 and tissue growth factor-β. These 'protective' cytokines may exaggerate the repair mechanism in response to epithelial damage, which in turn may lead to a fibrotic reaction that could promote BO. Further research on $CD4^+CD25^{high}$ and $CD4^+CD25^+$ cells in general as well as their respective cytokine productions will have to show if an imbalance in this cell type might be responsible for the ongoing fibrotic process found in BO [6-9]. To study these cells we will first assess their proposed interleukin-10 and tissue growth factor-β production by immunohistochemical staining and/or flowcytometric analysis for these cytokines on $CD4^+CD25^+$ cells as obtained by bronchoalveolar lavage at pre-set time points in patients after LTX [10].

BRONCHOALVEOLAR LAVAGE FLUID EXAMINATION - PROTEOMICS

As shown in table 3 of the introduction (chapter 1) of this thesis, numerous proteins have been measured in BALF with respect to BO. One of the new techniques that can help us to identify the total protein content of the epithelial lining fluid in patients with BO is two-dimensional electrophoresis. We will try, by using this 'proteomics' approach (a technology-based science which studies levels and post-translational modifications of a large number of proteins simultaneously, and their differences between healthy and diseased states) to characterise the protein composition in patients with BO and compare this to stable patients after LTX. Two-dimensional electrophoresis of BALF can identify large amounts (300-1200) so-called protein spots (each spot representing a different protein), which can be compared to a reference (e.g. healthy control) group. Once having located the differently expressed protein-spots, these spots can be individually selected and identified by mass spectrometry.

Within the next year we will start to measure BALF proteins after LTX with two-dimensional electrophoresis from patients with and without BO. This 'proteomic' approach will likely give us more insight into the complex cytokine changes that occur during the process of BO development as well as lead us to potential more specific therapeutic targets [11-14].

BRONCHOALVEOLAR LAVAGE FLUID – HO-1 EXPRESSION

To extend our results of chapter 10 we will measure HO-1 expression in patients after LTX in a more quantitative way. Therefore we have already developed a HO-1 flowcytometric analysis on alveolar macrophages derived from BALF samples. The next years we will apply this technique to longi-

tudinally obtained BALF samples, and study the potential role of HO-1 after LTX with respect to both BO and acute rejection.

THERAPEUTIC APPLICATION OF HO-1 AND CO AFTER LTX

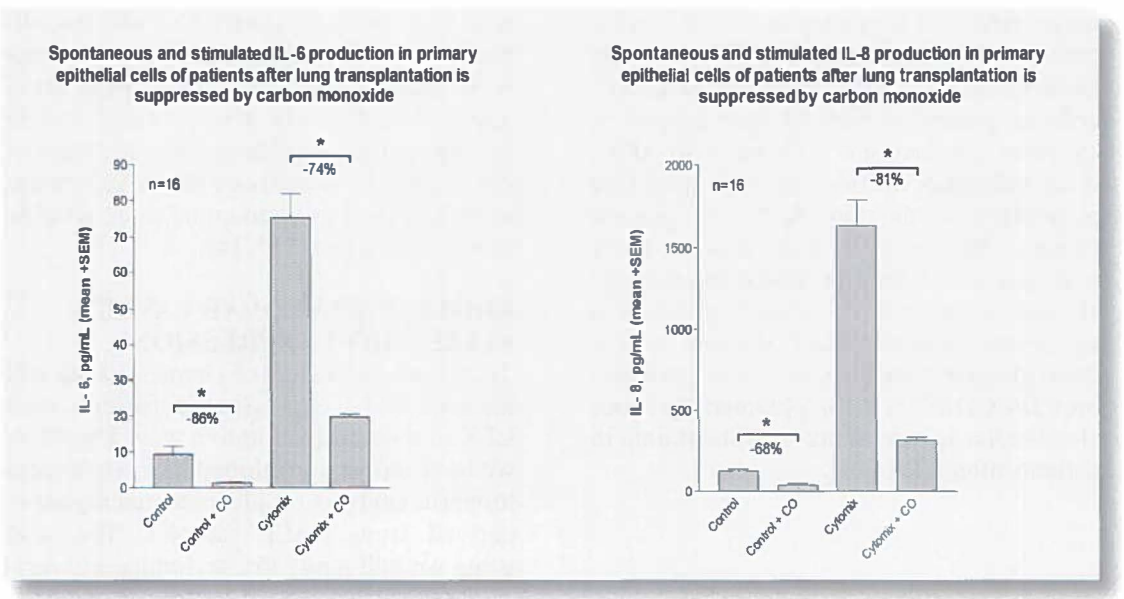
Therapeutically, we will focus the next years on the cytoprotective potentials of the heme oxygenase-1 and carbon monoxide system [15-17]. Preliminary data from our group showed that CO can suppress both spontaneously and 'cytomix'-induced IL-6 and IL-8 production in primary epithelial cells of patients after LTX (figure 1). Furthermore, in a pilot study we were able to show the protective effects of HO-1 induction on allograft rejection after rat-LTX. Administration of a single dosage of cobalt protoporphyrin-IX (a substrate that strongly induces HO-1) is already protecting the transplanted lung (figure 2). Our results clearly showed preservation of the alveolar space, with still present, but less intense perivascular infiltrates when compared to the non-treated animals. Thus based on the observation that administration of CO can reduce airway epithelial cell induced inflammation (figure 1), and that HO-1 induction can inhibit allograft rejection (figure 2), HO-1 and CO may have a potential important protective role in preserving allograft function both directly after LTX as well as on the longer run.

Based upon these preliminary results we will therefore examine:

- The mechanism by which both HO-1 and CO are able to modulate the inflammatory response of respiratory epithelial cells.
- The potential suppressive effect of HO-1 upregulation and CO exposure on allogenic T-cell induced respiratory epithelial cell inflammation [18].
- The most optimal modulation of the HO-1/CO system with regard to graft survival

FIGURE 1.

LTX/passage-2 primary epithelial cell cultures n=16, harvested by endobronchial brushing of patients after LTX, were in used in these experiments. After P2, when confluent, the CO-exposed cells were pre-treated for 2 hours with 250 ppm CO prior to stimulation with cytomix (IL-1β: 50U/ml plus TNFα: 100ng/ml plus LPS: 10ng/ml) in the presence or absence of 250 ppm CO (4 hrs). Four hours after stimulation the IL-6 and IL-8 levels were measured in the supernatants. All cells showed 75-85% viability as tested by trypan blue staining.



in a rat orthotopic LTX model. This, in advance to potential therapeutical human application of either HO-1 modulation and/or CO administration after LTX [19].

Our ultimate challenge is to apply the therapeutic potentials of HO-1 and CO to the treatment of patients after lung transplantation. *In vivo* models of liver and renal transplantation have shown that both HO-1 gene therapy and low-dose CO inhalation protects against allograft rejection [22-24]. The toxic properties of CO are well known in the field of pulmonary medicine. This invisible, odorless gas, still claims many victims on a yearly basis by accidental exposure. The toxic actions of CO relate to its high affinity for hemoglobin (240 fold greater than that of O_2). CO replaces O_2 rapidly from hemoglobin, causing tissue hypoxia [25-27]. At high concentrations, other mechanisms of CO-induced toxicity may include apoptosis, lipid peroxidation, and inhibition of drug metabolism and respiratory chain enzyme functions [26]. It has only recently has it become known that CO participates beneficially in many physiological reactions at very low concentrations. Where a CO exposure of 10,000 parts per million (ppm) (1% by volume CO in air) is toxic, 100-250 ppm (40-100 fold reduction) will stimulate the protective effects without apparent toxicity [28]. The potential use of inhaled CO as a clinical therapeutic modality is supported by the absence of cardiovascular and neurological side effects as recently reported while using a theoretically sufficient protective dosage of CO [29]. In the former study Hb-CO levels of

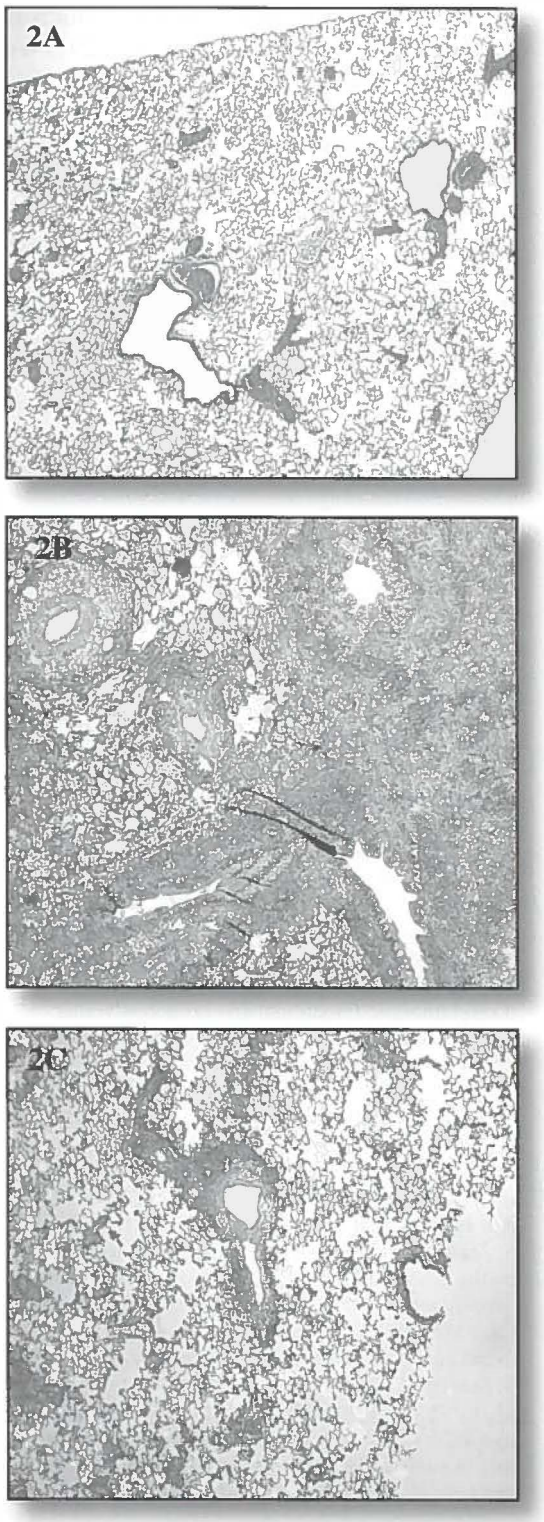


FIGURE 2A/2B/2C.
Cobalt protoporphyrin-IX treated Fisher to Wistar-Kyoto, orthotopic left lung transplantation) (lung tissue H&E staining, magnifications: A:10x B&C: 50x; A: no LTX, n=3; B: control LTX, n=3; C: cobalt protoporphyrin-IX treated LTX (resulting in heme oxygenase-1 up-regulation), n=3. Figures representative for n=3.

approximately 5% were reached, which is comparable to cigarette smoking [29]. We anticipate that the question whether CO can be used as an inhaled therapy for LTX will soon be replaced by questions of 'how much, how long, and how often?'. The fear of administration of CO must be weighed against the severe toxicity of the immunosuppressive agents as currently used, and the often negative outcome of solid organ transplantation despite the treatments available.

REFERENCES

1. Estenne M, Van Muylem A, Knoop C, Antoine M. Detection of obliterative bronchiolitis after lung transplantation by indexes of ventilation distribution. Am J Respir Crit Care Med. 2000;162:1047-51.
2. Siegel MJ, Bhalla S, Gutierrez FR, Hildebolt C, Sweet S. Post-lung transplantation bronchiolitis obliterans syndrome: usefulness of expiratory thin-section CT for diagnosis. Radiology. 2001;220: 455-62.
3. Reynaud-Gaubert M, Thomas P, Badier M, Cau P, Giudicelli R, Fuentes P. Early detection of airway involvement in obliterative bronchiolitis after lung transplantation. Functional and bronchoalveolar lavage cell findings. Am J Respir Crit Care Med. 2000;161:1924-9.
4. Gabbay E, Walters EH, Orsida B, Whitford H, Ward C, Kotsimbos TC et al. Post-lung transplant bronchiolitis obliterans syndrome (BOS) is characterized by increased exhaled nitric oxide levels and epithelial inducible nitric oxide synthase. Am J Respir Crit Care Med 2000;162:2182-2187.
5. Zayasu K, Sekizawa K, Okinaga S, Yamaya M, Ohrui T, Sasaki H. Increased carbon monoxide in exhaled air of asthmatic patients. Am J Respir Crit Care Med 1997;156:1140-1143.
6. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev. 2001;182:18-32.
7. Taylor PA, Noelle RJ, Blazar BR. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. J Exp Med. 2001;193:1311-8.
8. Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory CD25(+) T Cells in Human Kidney Transplant Recipients. J Am Soc Nephrol. 2003;14:1643-51.
9. F. Meloni, A. Fietta, L. Ciardelli, I. Mazzucchelli, E. Paschetto, A. Marone Bianco, M. Morosini, A. Cascina, P. Vitulo, E. Pozzi. CD4+CD25highCD69- T Cells in Lung Transplant Recipients: Do They Correlate with Tolerance? Am J Respir Crit Care Med 2003;167(7):A939
10. Oida T, Zhang X, Goto M, Hachimura S, Totsuka M, Kaminogawa S, Weiner HL. CD4+CD25- T cells that express latency-associated peptide on the surface suppress CD4+CD45RBhigh-induced colitis by a TGF-beta-dependent mechanism. J Immunol. 2003;170:2516-22.
11. Sabounchi-Schutt F, Astrom J, Hellman U, Eklund A, Grunewald J. Changes in bronchoalveolar lavage fluid proteins in sarcoidosis: a proteomics approach. Eur Respir J. 2003;21:414-20.
12. Noel-Georis I, Bernard A, Falmagne P, Wattiez R. Database of bronchoalveolar lavage fluid proteins. J Chromatogr B Analyt Technol Biomed Life Sci. 2002;771(1-2):221-36.
13. Magi B, Bini L, Perari MG, Fossi A, Sanchez JC, Hochstrasser D, Paesano S, Raggiaschi R, Santucci A, Pallini V, Rottoli P. Bronchoalveolar lavage fluid protein composition in patients with sarcoidosis and idiopathic pulmonary fibrosis: a two-dimensional electrophoretic study. Electrophoresis. 2002;23: 3434-44.
14. Reitsma PH, de Koster ChG. ['Proteomics': the mapping of all human proteins] Ned Tijdschr Geneeskd. 2003;147:99-104.
15. Morse D, Choi AM. Heme Oxygenase-1. The "emerging molecule" has arrived. Am J Respir Cell Mol Biol 2002;27:8-16
16. Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat.Med 1996; 2:87-90
17. Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. J Immunol. 2001;166:4185-94
18. Borger P, Kauffman HF, Scholma J, Timmerman JA, Koeter GH. Human allogeneic CD2+ lymphocytes activate airway-derived epithelial cells to produce interleukin-6 and interleukin-8. Possible role for the epithelium in chronic allograft rejection.

J Heart Lung Transplant. 2002;21:567-75.
19. Prop J, Ehrie MG, Crapo JD, Nieuwenhuis P, Wildevuur CR. Reimplantation response in isografted rat lungs. Analysis of causal factors. J Thorac Cardiovasc Surg 1984;87:702-11
20. Lee PJ, Alam J, Wiegand GW, Choi AM. Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia. Proc Natl Acad Sci U S A 1996;93:10393-8
21. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat.Med 2000;6:422-428.
22. Avihingsanon Y, Ma N, Csizmadia E, Wang C, Pavlakis M, Giraldo M, Strom TB, Soares MP, Ferran C: Expression of protective genes in human renal allografts: a regulatory response to injury associated with graft rejection. Transplantation 2002;73: 1079-1085.
23. Ke B, Shen XD, Melinek J, Gao F, Ritter T, Volk HD, Busuttil RW, Kupiec-Weglinski JW: Heme oxygenase-1 gene therapy: a novel immunomodulatory approach in liver allograft recipients? Transplant Proc 2001;33: 581-582.
24. Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry KR, Choi AM. Carbon Monoxide Induces Cytoprotection in Rat Orthotopic Lung Transplantation via Anti-Inflammatory and Anti-Apoptotic Effects. Am J Pathol. 2003;163:231-42.
25. Walker E, Hay A: Carbon monoxide poisoning. BMJ 1999;319:1082-1083.
26. Weaver, LK: Carbon monoxide poisoning. Crit Care Clin 1999;15:297-317.
27. Piantadosi CA: Carbon monoxide poisoning. N Engl J Med 2002;347:1054-1055.
28. Otterbein LE, Choi AM: Heme oxygenase: colors of defence against cellular stress. Am J Physiol Lung Cell Mol Physiol 2000, 279:L1029-L1037.
29. Zevin S, Saunders S, Gourlay SG, Jacob P, Benowtitz NL: Cardiovascular effects of carbon monoxide and cigarette smoking. J Am Col Cardiol 2001;38:1633-1638.

NEDERLANDSE SAMENVATTING VOOR DE NIET-MEDISCH GESCHOOLDEN

foto + bewerking: Marijke de Haan

Diverse longziekten zoals de erfelijke alfa-1 antitrypsine deficiëntie en cystic fibrosis (taaislijm ziekte), longemfyseem, long fibrose, primaire en secundaire pulmonale hypertensie en een aantal nog zeldzamere longaandoeningen kunnen reeds op jonge leeftijd tot ernstige invaliditeit en sterfte leiden. Op het moment dat één van deze ziekten het eindstadium heeft bereikt is longtransplantatie de enige vorm van behandeling die nog beschikbaar is voor deze patiënten. Jaarlijks ondergaan in Nederland ongeveer 35 mensen een longtransplantatie, en wereldwijd zijn er de afgelopen 15 jaar ongeveer 15.000 patiënten getransplanteerd.
Na de transplantatie blijft een longtransplantatiepatiënt echter een zeer onzekere toekomst houden. Daar waar een 'nier' na de transplantatie gemiddeld 10 jaar goed functioneert en de patiënt, als de nier ophoudt met werken daarna in veel gevallen weer worden gedialyseerd, overlijdt de helft van de longtransplantatiepatiënten binnen de eerste 5 jaar na de transplantatie.

In de eerste zes maanden na de transplantatie overlijdt al één op de tien ontvangers van een getransplanteerde long. Na een succesvolle transplantatie zijn het vooral infecties in de longen en elders in het lichaam, ernstige nierbeschadiging, het sneller ontstaan van kanker, suikerziekte en door virusinfecties veroorzaakte lymfklierkanker stuk voor stuk voor elke patiënt een continue bedreiging. Het grootste probleem na longtransplantatie is echter het ontstaan van chronische afstoting van de getransplanteerde longen. Dit proces van chronische afstoting van de longen wordt 'bronchiolitis obliterans' genoemd. Deze ziekte kenmerkt zich door een steeds verdere beperking van de longfunctie. Dit heeft als gevolg dat de patiënt veel activiteiten door kortademigheid niet meer kan doen en de long erg gevoelig wordt voor infecties. Uiteindelijk is bronchiolitis obliterans verantwoordelijk voor het overlijden van meer dan de helft van de getransplanteerde patiënten.

Bronchiolitis obliterans ontstaat meestal na het eerste jaar volgend op de longtransplantatie, maar het is niet bekend hoe bronchiolitis obliterans ontstaat. Uit verschillende onderzoeken is gebleken dat veel factoren risico's zijn voor het ontstaan van bronchiolitis obliterans na longtransplantatie: de longen van een 'oudere' donor, een lange tijdsduur tussen de uitname van het orgaan en de transplantatie zelf, een groter aantal acute afstotingsreacties van de longen, virusinfecties, chronische luchtweginfecties, een groot verschil in weefseltypering tussen de donor en ontvanger van de longen en wellicht zelfs de medicijnen die worden gebruikt om de afstoting van de longen te voorkomen.

Door al deze schadelijke 'prikkels' voor de 'nieuwe' longen ontstaat een chronische beschadiging aan de bloedvaten- en vooral de kleinere luchtwegen in de long. Na beschadiging proberen weefselcellen in de long de schade te repareren. Als deze reparatie-reactie overdreven sterk verloopt ontstaat er een ophoping van dode cellen en littekenweefsel in de kleinere luchtwegen van de long, die uiteindelijk hierdoor volledig worden afgesloten. Door deze afsluiting kan er geen lucht meer van en naar het stukje long achter die afsluiting en werkt dus vervolgens niet meer mee aan de ademhaling. Uiteindelijk veroorzaakt dit proces een groot verlies van longfunctie.
Het grote probleem van het ontstaan van bronchiolitis obliterans is dat het moeilijk op tijd te ontdekken is en dat er geen goede behandeling voor beschikbaar is. Op dit moment wordt aan de hand van het meten van de uitademingsnelheid van de lucht (longfunctie) vastgesteld hoe het met de getransplanteerde longen gaat. Op het moment dat deze uitademingsnelheid blijvend meer dan 20% achteruit is gegaan, is we-

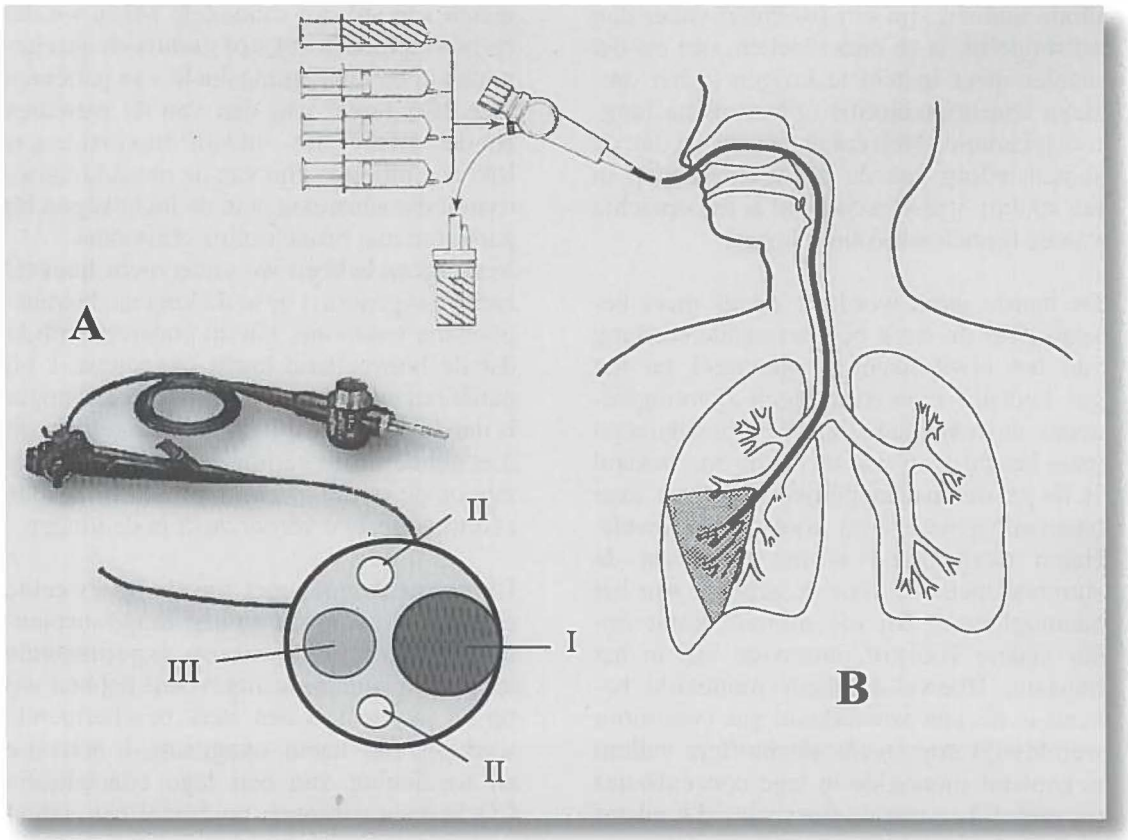
reldwijd afgesproken dat er dan sprake is van het 'bronchiolitis obliterans syndroom' of kortweg 'BOS'.

Dit proefschrift is geschreven omdat het op dit moment niet goed mogelijk is bronchiolitis obliterans vast te stellen, omdat het niet bekend is hoe bronchiolitis obliterans ontstaat en omdat er eigenlijk geen goede behandeling voor beschikbaar is. Na een algemene inleiding over het ontstaan, het vaststellen en behandelen van bronchiolitis obliterans, behandelt dit proefschrift in het eerste deel het onderzoek naar de ontstekingsreactie in de luchtwegen na longtransplantatie en in deel twee de mogelijke toekomstige toepassing van een nieuwe therapie tegen afstoting na transplantatie.

Om de mate van ontsteking in de long te kunnen onderzoeken wordt er vaak gebruik gemaakt van de bronchoalveolaire lavage. De bronchoalveolaire lavage is een techniek waarbij een klein gedeelte van de long wordt gespoeld met fysiologische zoutoplossing en onderzocht kan worden op allerlei cellen en eiwitten die in dat vocht mee terug zijn gezogen. Om een bronchoalveolaire lavage te kunnen uitvoeren is een bronchoscopie nodig. Dit is een flexibele dunne slang met een camera en een werkkanaal waardoor het vocht kan worden ingespoten en teruggezogen. In figuur 1 is te zien hoe een bronchoalveolaire lavage wordt uitgevoerd.
Er is tot nu toe al veel onderzoek gedaan met behulp van de bronchoalveolaire lavage om meer inzicht te krijgen in bronchiolitis obliterans na longtransplantatie. Hierbij worden ontstekingscellen en eiwitten bepaald in de bronchoalveolaire lavage vloeistof die is teruggezogen uit de patiënt. Er is echter tot nu toe nog nooit onderzocht wat de normale situatie in de longen is van deze cellen en eiwitten na een longtransplantatie. Daarom zijn we begonnen met het onderzoeken van de normale hoeveelheid en de onderlinge verdeling van de verschillende ontstekingscellen in de longen na longtransplantatie. Hierbij blijkt vooral dat deze erg verschilt van patiënten zonder longtransplantatie, waarschijnlijk als gevolg van het gebruik van medicijnen om afstoting van de longen te voorkomen. Daarnaast veranderen in de eerste twee jaar na de transplantatie de verhoudingen van deze ontstekingscellen sterk.
Na het vaststellen van de normale verdeling van de ontstekingscellen in de long na transplantatie, zijn we gaan onderzoeken hoe afwijkend die verdeling van ontstekingscellen is en welke eiwitten met name aanwezig zijn als er bronchiolitis obliterans aanwezig is in de longen. Het blijkt dat met name het percentage neutrofiele granulocyten (een ontstekingscel) en de hoeveelheid interleukine-8 (een eiwit dat een ontstekingreactie door de neutrofiele granulocyt stimuleert) sterk verhoogd kan zijn bij patiënten met bronchiolitis obliterans. Het uitvoeren van een bronchoalveolaire lavage na longtransplantatie kan dus bijdragen aan het opsporen van bronchiolitis obliterans. Nadat we onderzocht hadden welke cellen en eiwitten kenmerkend waren als er eenmaal chronische afstoting in de longen aanwezig was, zijn we gaan onderzoeken of er in die bronchoalveolaire lavage ook cellen en eiwitten te vinden waren die voorspellend zijn of een patiënt op een later tijdstip een chronische afstoting zal krijgen. Vervolgens laten we zien dat in de eerste bronchoalveolaire lavage na longtransplantatie met name de aanwezigheid van de eosinofiele granulocyt en een sterk verhoogd interleukine-6 gehalte in grote mate voorspellend zijn voor het ontstaan van bronchiolitis obliterans op een later moment. Na het vaststellen van de normaalwaarden in de bronchoalveolaire lavage (na longtransplantatie), na deze vervolgens te hebben onderzocht in bronchiolitis obliterans en de voorspellende factoren voor het ontstaan van bronchiolitis obliterans in de bronchoalveolaire lavage te heb-

ben onderzocht, zijn we gaan onderzoeken welke invloed de medicijnen, die de patiënten gebruiken om te voorkomen dat de long word afgestoten, hebben op de aanwezigheid van de diverse ontstekingscellen in de getransplanteerde longen. De laatste jaren zijn er een groot aantal nieuwe medicijnen beschikbaar gekomen om afstoting van organen na transplantatie te voorkomen. Van deze medicijnen wordt veronderstelt dat ze beter werken, maar dat is tot nu toe nog niet goed onderzocht. Uit ons onderzoek blijkt dat in de eerste maand na longtransplantatie de ontstekingscellen in de longen erg verschillen wanneer we patiënten onderzoeken die gebruik maken van de 'traditionele' en met de 'nieuwe' medicijnen. Theoretisch lijkt het gebruik van de nieuwe medicijnen tot een betere verhouding van de verschillende ontstekingscellen te leiden. Deze betere verhouding zou in theorie kunnen leiden tot minder infecties en minder afstotingsreacties na longtransplantatie. Om te laten zien dat het gebruik van de bronchoalveolaire lavage als onderzoeksmethode niet alleen voor wetenschappelijk gebruik nuttig is, laten we tevens zien dat we met deze methode ook andere ziekten na longtransplantatie kunnen vaststellen. Bij een patiënt die getransplanteerd is vanwege sarcoïdose in

FIGUUR 1.
De bronchoalveolaire lavage. Een klein gedeelte van de long wordt gespoeld (A) met behulp van een bronchoscoop (B). De ingespoten hoeveelheid fysiologisch zout (meestal 3-4x 50ml) wordt via de bronchoscoop teruggezogen en opgevangen in een verzamelpot. I-III: dwarsdoorsnede van de bronchoscoop; I: het 'werkkanaal'; II: 'de lichtbronnen'; III: de camera.



de longen, waren we in staat door middel van het gebruik van de bronchoalveolaire lavage de sarcoïdose opnieuw vaststellen in de getransplanteerde long. Omdat het uitvoeren van de bronchoalveolaire lavage toch een redelijk belastend onderzoek voor de patiënt is, hebben we ook onderzocht of de minder belastende methode van 'sputuminductie' geschikt zou zijn om uit te voeren bij patiënten na longtransplantatie.

Naast de bronchoalveolaire lavage is ook sputuminductie een methode om materiaal (met name cellen) uit de luchtwegen te verkrijgen en wordt als zodanig regelmatig gebruikt bij het wetenschappelijk onderzoek naar astma. We hebben laten zien dat deze methode in 93% succesvol is bij patiënten die goed hersteld zijn van de transplantatie, en dat zonder noemenswaardige bijwerkingen. Sputuminductie zou daarom een methode kunnen zijn om patiënten vaker dan nu mogelijk is te onderzoeken, om op die manier meer inzicht te krijgen in het ontstaan van bronchiolitis obliterans na longtransplantatie. Het enige nadeel is dat de samenstelling van de ontstekingscellen in het sputum wel verschillend is ten opzichte van de bronchoalveolaire lavage.

De laatste jaren wordt er steeds meer bekend over de sterk beschermende werking van het eiwit haem oxygenase-1 en het gas koolstof monoxide tegen afstotingsreacties na transplantatie. We zijn allereerst gaan beschrijven wat er tot nu toe bekend is in de wetenschappelijke literatuur over haem oxygenase-1 en koolstof monoxide. Haem oxygenase-1 is het eiwit dat de sturende factor is voor de afbraak van het haemoglobine. Bij die afbraak komt onder andere koolstof monoxide vrij in het lichaam. Hoewel koolstof monoxide bekend is als een verstikkend gas (waardoor wereldwijd nog steeds slachtoffers vallen) is koolstof monoxide in lage concentraties (te vergelijken met de hoeveelheid koolstof monoxide die de ingeademde rook van 10-15 sigaretten per dag bevat) een molecuul dat een beschermende werking heeft bij ontstekingsreacties zoals de afstoting van getransplanteerde organen.
In theorie kan een behandeling gericht op het verhogen van de activiteit van haem oxygenase-1 in het lichaam, of het inademen van een lage concentratie koolstof monoxide wellicht de afstotingsreactie na transplantatie voorkomen. Om in eerste instantie te beoordelen of zowel het haem oxygenase-1 als ook het koolstof monoxide een rol speelt bij het ontstaan van bronchiolitis obliterans hebben we het gehalte aan koolstof monoxide gemeten in de uitgeademde lucht van patiënten na longtransplantatie. Bij de meting van koolstof monoxide hebben wij geen verschil kunnen aantonen tussen patiënten met en zonder bronchiolitis obliterans. Bij het gelijktijdig meten van stikstof monoxide zagen we dat de hoeveelheden stikstof monoxide-gas gemeten in de uitademingslucht van patiënten met BOS hoger was dan van de patiënten zonder BOS. Het stikstof monoxide-gas lijkt een uiting te zijn van de ontstekingsactiviteit die aanwezig is in de luchtwegen bij patiënten met bronchiolitis obliterans.
Vervolgens hebben we onderzocht hoeveel haem oxygenase-1 er in de long na de transplantatie voorkomt. Uit dit onderzoek blijkt dat de hoeveelheid haem oxygenase-1 bij patiënten met bronchiolitis obliterans hoger is dan bij longtransplantatiepatiënten die dit niet hebben. Die verhoging kan een reactie zijn op de sterke prikkels die de chronische afstotingsreactie veroorzaakt in de longen.

Uit vooronderzoek met zowel opgekweekte cellen van patiënten na een longtransplantatie als met ratten waarin experimentele longtransplantatie is uitgevoerd hebben wij op dit moment al een sterk beschermende werking van haem oxygenase-1 activatie en toediening van een lage concentratie CO kunnen aantonen op zowel een aantal

ontstekingsreacties als op de afstoting van getransplanteerde longen in de rat.

Samengevat is bronchiolitis obliterans na longtransplantatie een zeer ernstige aandoening. Bij het ontstaan ervan zijn zeer verschillende factoren van invloed. Al deze factoren veroorzaken een ontstekingsreactie in de long, die uiteindelijk tot het ontstaan van volledig afgesloten kleine luchtwegen leidt. Met behulp van bronchoalveolaire lavage hebben we meer inzicht in de aanwezige ontstekingsreactie bij bronchiolitis obliterans gekregen en we hopen in de komende jaren met nieuwe analysetechnieken dit inzicht verder te verfijnen. Met name gezien de nog steeds zeer sombere levensverwachting van patiënten na longtransplantatie lijkt behandeling met het verhogen van de haem oxygenase-1 activiteit in het lichaam, dan wel met het toepassen van lage concentraties koolstof monoxide een toekomstige reële behandelingsmogelijkheid in de transplantatiegeneeskunde.

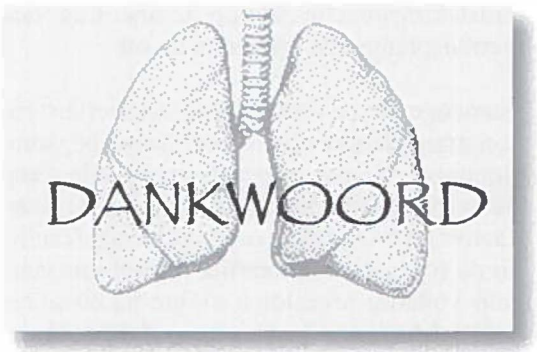


foto + bewerking: Marijke de Haan

DANKWOORD

Het proefschrift en de opleiding zijn klaar, maar het werk moet eigenlijk nog beginnen. Toch is dit een mooie gelegenheid om iedereen, die mij de afgelopen jaren in staat heeft gesteld met veel plezier in Groningen te werken aan zowel mijn opleiding tot longarts en dit proefschrift, te bedanken *Gerard en Dirkje,* bedankt voor het vertrouwen dat jullie zowel in het verleden als naar de toekomst in mij hebben uitgesproken, voor jullie samenwerking als promotores van dit proefschrift, voor de vrijheid die ik bij het maken ervan heb gekregen en voor de support voor de ontwikkeling van mijn andere ideeën.

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Nogmaals aan iedereen:
Mijn dank is groot!
En we gaan gewoon weer verder...

Dirk-Jan

nov 2003